

**THE IMPACT OF NEONATAL NUTRITION ON THE GROWTH, FERTILITY,
HEALTH, MILK YIELD AND DAY OLD CALF PERFORMANCES OF HOLSTEIN
DAIRY HEIFERS**

**Thesis submitted in accordance with the requirements of the University of Liverpool for
the degree of Doctor of Philosophy**

By

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October 2017

DECLARATION

Unless otherwise acknowledged, this thesis is all my own work, carried out in the Institute of Infection and Global Health, University of Liverpool, Veterinary Field Station, Neston, South Wirral, United Kingdom under the supervision of Professor Robert Frank Smith. No part of this thesis, in any form has been submitted to any other university for any other degree.

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ABSTRACT

The impact of neonatal nutrition on the growth, fertility, health, milk yield and day old calf performances of Holstein dairy heifers.

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Neonatal nutrition may influence performance in later life in many species; however, there have been a few studies of this phenomenon in cattle. Determining if there are direct and residual effects of nutrition during the first 60 days of life may help optimise lifetime productivity. Underfeeding reduces calf weaning weight and delays puberty, which reduces the potential lifetime productivity of the dairy heifers. As well as repercussions on calf health, growth and welfare; many studies in human suggest that underfeeding of the newborn is a major risk factor for metabolic diseases in the adult.

The aims of this study were to investigate the performances of Holstein heifers that were fed increased milk replacer compared to restricted volumes during their early life and to determine the impact of this on key performance indicators of these animals as future potential the best cows in the herd. Thus, a total of eighty eight heifers were included in this study, which forty seven heifers were in Group A (*ad libitum* milk replacer fed group) and forty one heifers were in Group R (restricted milk replacer fed group). They were enrolled 8 weeks prior to predicted calving based on artificial insemination dates of pregnancies confirmed by per rectum ultrasound examination of the uterus and 282 days of gestation period. The body weight, body condition score, withers and loin height, heart and belly girth, crown to rump length and hock fetlock length were recorded from 8 weeks prior to predicted calving until 30 weeks of postpartum. Blood samples were collected for β -hydroxybutyrate (BHB) concentration measurements and milk samples were collected for pregnane profiles analysis. Data on physical measurements (heifers and calves), pregnane profiles, reproductive parameters, BHB concentration values, health parameters, milk yield and milk components were analysed. The MilkBot parameters were used to describe the lactation curve between the 2 groups.

There was no large effect of different pre-weaning feeding strategies during early life of heifers on their growth, fertility, health, milk yield and day old calf performances between Group A and Group R during first lactation period. There were no differences in MilkBot parameters; estimated scale, decay, persistence, peak milk and peak day between the 2 groups. However, several significant findings were observed; heifers in Group A had higher milk protein percentage and higher in somatic cell counts (SCC). Meanwhile, heifers in Group R had higher number of delayed ovulation type 1 (DOV1) profile, higher incidence of subclinical ketosis (SCK) and higher in estimated ramp. Another finding was heifers produced more milk following birth of a bull calf regardless of groups.

ACKNOWLEDGEMENTS

Firstly, I would like to thank to the Government of Malaysia for Federal Government Scholarship (Hadiah Latihan Persekutuan, HLP) especially to the Director General of Public Service Department and Director General, Department of Veterinary Services for a scholarship and study leave. Without HLP financial support, this study would not have been accomplished.

Huge thanks go to my supervisors Professor Robert Frank Smith and Dr Dai Grove-White for their support and encouragement. I have been so lucky to have had two amazingly dedicated supervisors. They have offered masses of support, guide and advice throughout my study. Thanks also to Dr Peter Cripps for his assistance and advice in statistic at the beginning of my study before his retirement.

Many thanks are due to Dave Jones and Jenny Fick, who helped me herd numerous lively heifers from the yard every Thursday morning to the weigh scales and back and took blood samples. Jean Routly deserves a thank you for helping me in the Wellcome Building especially during enzyme immunoassays (EIA) session at the beginning.

I would also like to thank John Cameron, Andrew Parkinson and Woodpark Farm staff for the use of facilities and animals for the study.

Thanks must also go to Ehrlich (DairySight LLC, Argyle, New York) for the MilkBot lactation model.

Finally, I would like to thank my wife: Noreyah Ahmad and my children: Nur Aina Naqiyah, Nur Qistina Batrisyia, Danish Qayyum and Nur Qaysha Shofiyyah; for their moral support and understanding during my study.

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ABBREVIATIONS

ADG	Average daily gain
AFC	Age at first calving
AI	Artificial insemination
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BCS	Body condition score
BG	Belly girth
BHB	β -hydroxybutyrate
BP	Best prediction
BW	Body weight
CIS	Cattle Information Service
CK	Clinical ketosis
CL	Corpus luteum
CLA	Commencement of luteal activity
CRL	Crown rump length
DA	Displaced abomasum
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
DOV	Delayed ovulation
EIA	Enzyme immunoassays
eRDP	Effective rumen-degradable protein

FSH	Follicle stimulating hormone
GnRH	Gonadotrophin releasing hormone
HFL	Hock fetlock length
HG	Heart girth
ICC	Intra class correlation
IGF-1	Insulin like growth factor 1
ILI	Inter luteal interval
IQR	Interquartile range
KPI	Key performance indicators
LH	Luteinizing hormone
LP	Luteal phase
MB	MilkBot lactation model
ME	Metabolisable energy
NDF	Neutral detergent fibre
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NM	Null model
PCL	Persistent corpus luteum
PNC	Postnatal check
RF	Rumen fill
RFM	Retained foetal membrane
SCC	Somatic cell counts
SCK	Subclinical ketosis
TAG	Triacylglycerides
TMR	Total mixed ration

VFA	Volatile fatty acids
VLDL	Very-low-density-lipoproteins
WH	Withers height
γGT	γ -glutamyltransferase

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Chapter 1

Introduction and Literature Review

1.1 Introduction

In our dairy industry, young animals are often used for breeding before their body growth is complete, which results in competition for nutrients between the growing animal and the developing foetus. The main factors influencing nutrient partitioning between the dam and foetus include age of the dam, number of foetuses, production demand and environmental stress (Reynolds et al., 2010). Foetal programming affects neonatal mortality and morbidity, postnatal growth rate, body composition, health and reproduction (Wu et al., 2006). Birth weights of calves born by still growing heifers are reduced depending on the dam's age and body weight in comparison with non-growing and heavier older cows (Holland and Odde, 1992). At the beginning of pregnancy, maternal nutrient restriction can lead to intrauterine growth retardation of the foetus (Robinson et al., 1999). Intrauterine growth retardation alters placental function, foetal growth and development. This predisposes offspring to cardiovascular, metabolic and endocrine diseases in later life in humans, rodents and pigs (Simmons et al., 2001; Bee, 2004; Gluckman and Hanson, 2004).

Several studies have examined the long term effects of pre-weaning nutrition on dairy heifers' milk yield (Bar-Peled et al., 1997; Shamay et al., 2005; Davis-Rincker et al., 2006; Moallem et al., 2006; Aikman et al., 2007; Drackley et al., 2007; Morrison et al., 2009; Raeth-Knight et al., 2009; Terre et al., 2009). Unfortunately, there is a lack of studies on long term effects of pre-weaning nutrition on growth, fertility, health and day old calf performances of the heifers. Therefore, this study was designed to further investigate the long term effect of early life nutrition on growth, fertility, health, milk yield and day old calf performances of the heifers during their first lactation.

The first objective of this study was to evaluate the effect of different milk replacer feeding strategy in early postnatal life of heifer calves on their future growth and also day old calf

performance by comparing their performance between *ad libitum* milk replacer fed calves (Group A) and restricted milk replacer fed calves (Group R) groups, (Chapter 3).

The second objective of this study was to evaluate the effect of different milk replacer feeding strategy in early life of heifer calves on their future fertility and health performance using the same cohort of animals, (Chapter 4).

The third objective of this study was to evaluate the effect of different milk replacer feeding strategy in early life of heifer calves on their first lactation performance including the effect of type of calf on milk yield, (Chapter 5).

Finally an overview of the findings in context of the UK and global dairy industries will be given (Chapter 6).

1.2 Literature Review

1.2.1 Pre-Weaning Heifer Calf Feeding Programme

Milk and milk replacer feeding strategies for young animals have been studied for many years. Early studies focused on levels of milk and milk replacer feeding after the concept of using milk replacers had gained popularity. The consequences later in life, both during the growing and the productive period of feed sources and feed level have been studied in many species of animals. Current feeding recommendations suggest that calves are fed 10 to 12% of their birth body weight in milk; an amount that translates to between 4 and 5 kg/day of liquid milk and is less than 50% of reported *ad libitum* intakes of suckling calves (Appleby, 2001; Tedeschi and Fox, 2009). Calves fed restricted quantities of milk consume the entire amount of milk rapidly at the time of delivery and have frequent unrewarded visits to the milk feeder, suggesting that they are hungry and they may experience poor welfare (De Paula Vieira et al., 2008). Moreover, when fed via an artificial teat, restricted fed calves spend considerable amounts of time engaged in non-nutritive sucking throughout the day.

In contrast to conventional restricted milk feeding, enhanced milk feeding programmes provide elevated quantities of milk or *ad libitum* access to milk. Meal frequencies similar to suckling calves have been reported in *ad libitum* milk feeding systems, average meal frequency ranging from 4 to 10 meals per day (Appleby, 2001; De Paula Vieira et al., 2008). In general, providing greater quantities of milk appears to be beneficial in allowing preferred feeding patterns and supporting increased rate of weight gain and greater structural growth, approximately 1.0 kg/day on enhanced milk feeding programmes *versus* 0.45 kg/day on conventional restricted milk feeding programmes as reviewed by Khan et al., (2011). However, enhanced milk feeding programmes may present challenges at time of weaning, if the calf has not begun consuming sufficient amounts of solid feed prior to removal of milk. When provided greater quantities of milk, calves have less frequent and smaller meals of

concentrate (Miller-Cushon et al., 2013), indirectly delaying rumen development (Hill et al., 2008).

There are many studies in various animal species that demonstrate early life nutrient status has long term developmental effects. Beside improvement in immune system, there appear to be other factors that are impacted by early life nutrient status. A study conducted by Ballard et al. (2005), reported that at 200 days in milk, calves that were fed milk replacer at approximately twice normal feeding rates produced 694 L (1,543 pounds) milk more than the calves that were received 1 pound of milk replacer powder per day. Calving age in this study was not affected.

Feeding different amounts of milk has the potential to influence the future of calf, as there is evidence that high early growth rates are associated with increased milk production into the first lactation (Soberon and Van Amburgh, 2013). The amount of milk feed may have long term metabolic consequences for the calf, infrequent milk meals (3.5 L feedings twice daily) have been found to increase serum insulin to glucose ratio (Terre et al., 2009), suggesting that this type of feeding may elicit insulin resistance (Bach, 2012). Findings in studies of Shamay et al. (2005) and Moallem et al. (2010) were significant, specifically because they suggested that milk replacer quality was important to achieve the milk response, as was protein status of the animal post weaning. In these studies, the calves were fed 23% crude protein (CP), 12% fat and milk replacer containing some soy protein or whole milk. Furthermore, post-weaning calves were fed similarly until 150 days and the diets were protein deficient (13.5% CP). Starting from 150 days, calves from both pre-weaning treatments were supplemented with 2% fish meal until 300 days of life. Calves that allowed to consume the whole milk (*ad libitum* for 60 minutes) and supplemented with additional protein produced approximately 765 L (1,700 pounds) more milk in the first lactation; indicating that early life response could be muted by inadequate protein intake during post-weaning.

1.2.2 Nutritional Effects from Birth to Weaning

Previous studies comparing of heifer calves fed either restricted amounts of milk replacer or allowed to suckle a cow before weaning, showed that suckling calves had higher body weight gains, resulted a production of more milk in their first lactation because of higher nutrient intake before weaning (Bar-Peled et al., 1997; Drackley, 2005). Increasing protein and energy intake through milk replacer in Holstein heifer calves from 2 to 8 weeks of age can increase the rate of mammary parenchyma development (Brown et al., 2005). However, if this increase would translate into higher milk production later in life was not reported.

Heifer calves consuming whole milk for body weight gains of 1.1 kg/day tended to produce more milk as cows than did heifer calves fed a restricted amount of whole milk before weaning (Foldager and Krohn, 1994). In support of the above view, Israeli Holstein heifer calves reared by *ad libitum* milk consumption for the first 50 days of life as compared with milk replacer fed heifer calves had higher body weight, decreased onset of puberty by 23 days and increased fat corrected first lactation milk yield (Shamay et al., 2005). Whole milk fed calves had higher body weight and average daily gain (ADG) at weaning, lower age at first insemination and lower age at pregnancy and at calving compared to milk replacer fed heifers. The first lactation milk yield and 4% fat corrected milk yield were higher for whole milk fed heifers than milk replacer fed heifers (Moallem et al., 2010).

Greater body weight gain, structural growth and more physically developed rumen were observed in Holstein calves on a step down method compared with a conventional flat rate method (Khan et al., 2007). This method could be used as a mean to achieve faster growth rate and early puberty in Holstein calves. *Ad libitum* intake of milk by dairy calves through an artificial nipple can allow for increased milk intake and weight gain with no detrimental effects on intake of solid food after weaning when compared with milk feeding by bucket twice daily at 10% of body weight (Jasper and Weary, 2002).

1.2.3 Mammary Gland Growth

Mammary gland growth is a major determinant of milk yield capacity and longevity of lactation. It starts from foetal stage and the basic structures are formed in foetal life and the outer shape of the glands is fully developed at birth, but epithelial cells are rudimentary (Sinha and Tucker, 1969). At first month after birth, the mammary ducts and fat pads grow rapidly, but no alveoli are formed (Purup et al., 1993). In contrast, the non-epithelial tissues for example the stroma and the circulatory system are almost fully developed at birth. In the first few months after birth the glands grow at the same rate as the rest of the body (isometric growth). Only the non-epithelial tissue grows during this period.

At 2 to 3 months of age, the glands start to grow at a faster rate than the rest of the body (allometric growth) (Sinha and Tucker, 1969). In this phase there is rapid growth of the fat pad and the ducts that branch into it. There are still no alveoli formed. The ducts need the fat pad for their growth (Faulkin and DeOme, 1960) and are surrounded by connective tissue (Woodward et al., 1993).

The most rapid pre-pubertal mammary growth relative to body growth occurs from 3 to 9 months of age at body weight between 90 and 230 kg and is the critical period during which the mammary growth of a Holstein heifer can be influenced by the nutrition (Tucker, 1987). Many studies suggest that the allometric growth phase ends at onset of puberty or shortly thereafter (Sinha and Tucker, 1969; Sejrsen et al., 1982). At this stage of development, the mammary glands of heifers weigh about 2 to 3 kg, of which only 0.5 to 1 kg is parenchyma. The parenchyma usually contains about 10 to 20% epithelial cells, 40 to 50% connective tissue and 30 to 40% fat cells (Sejrsen et al., 1982). In comparison, lactating mammary glands can weigh approximately 25 kg (Foldager and Sejrsen, 1991) and lactating parenchyma consists of 40 to 50% epithelial cells (ducts and alveoli), 15 to 20% lumen, 40% connective tissue and almost no fat cells (Harrison et al., 1983).

The allometric growth phase of the mammary glands is closely related to the development of the reproductive systems. For instance, ovariectomy in the first week of life abolishes mammary growth due to a removal of oestrogen secreted by the ovaries. It has been shown that oestrogen replacement can restore mammary growth in heifers (Wallace, 1953). Polyunsaturated fat has been shown to increase pubertal mammary growth in sheep (McFaden et al., 1990).

1.2.4 Effect of Pre-Pubertal Nutrition on Mammary Growth

Studies have demonstrated a reduced amount of parenchyma tissue and a lowered future milk yield after high feeding level in the pre-pubertal period in both dairy and beef heifers (Harrison et al., 1983; Buskirk et al., 1996; Sejrsen et al., 2000). Increasing energy intake of pre-pubertal heifers (age 11 weeks; body weight 107 kg) for different durations (0, 3, 6 or 12 weeks); decreased the percentage of mammary epithelial cells in terminal ductal area, decreased the mass of fat free parenchyma per unit of carcass and increased the mass of mammary fat proving negative effects of high energy diets on mammary parenchymal mass at puberty (Davis Rincher et al., 2008). Thus, feeding high energy diets hastens puberty but mammary growth is not proportional to body growth and reduces mammary parenchymal tissue. In contrast, Daniels et al. (2009) reported that when Holstein heifers were fed varying amounts of the same diet to achieve 2 rates of average daily gain (ADG) 650 or 950 g/day and then slaughtered at body weight of 100, 150, 200, 250, 300 or 350 kg to study mammary parenchymal development; a higher rate of gain had no effect on mammary parenchyma and had no negative impact on ductal development. On the other hand, the number of epithelial and luminal structures present in mammary parenchyma increased with increasing body weight. Increasing pre-pubertal body weight gains can have a negative impact on mammary parenchyma cell numbers (Meyer et al., 2006a) and first lactation milk yield (Radcliff et al.,

2000). During pre-pubertal allometric phase of growth the mammary parenchymal mass, deoxyribonucleic acid (DNA) content or both are reduced in heifers reared on an elevated level of nutrient intake (Capuco et al., 1995). In contrast, Meyer et al. (2006b) found that basal proliferation of pre-pubertal bovine mammary epithelial cells was not reduced by feed intake to achieve 950 g/day body weight gain. The level of nutrient intake especially of dietary protein had minimal influence on mammary epithelial cell proliferation, the rate of parenchyma DNA accretion and the total parenchyma DNA (Meyer et al., 2006b). However, mammary fat pads were directly influenced by elevated energy and protein intake (Meyer et al., 2006a).

Efforts to correlate pre-pubertal mammary gland development with future productivity have been unsuccessful and this might be explained by the normal development of the mammary gland. In heifers, the epithelium of the mammary gland is composed exclusively of ducts and stoma tissue. Secretory cells are not present in the mammary gland of non-pregnant mammals and nutrient intake did not have an effect on epithelium growth from weaning to puberty. It is during pregnancy that all of the secretory tissue differentiates and the alveolar structures form. Thus, changes in the proliferation or total mass of the mammary parenchymal tissue pre-breeding, fail to correlate with milk production (Waldo et al., 1998; Radcliff et al., 2000; Smith, 2002). It is now clear that pre-weaning is the stage of life where mammary epithelium is responsive to nutrient intake (Meyers et al., 2006b; Brown et al., 2005a). Several studies that focused on the correlation of nutrient intake or average daily gain (ADG) pre-weaning, with respect to future milk production have shown significant milk yield increases with increased ADG prior to weaning.

Decrease in first lactation milk yield is associated with high energy diets fed during the pre-pubertal period has been attributed to reduced growth of mammary parenchyma and a concurrent increase in the deposition of mammary adipose tissue (Swanson, 1960).

1.2.5 Effect of Pre-Pubertal Nutritional Composition on Mammary Growth and Milk Yield

Several studies have reported an influence of nutritional composition on mammary growth and subsequent milk yield. Capuco et al. (1995) documented that total mammary parenchymal DNA and ribonucleic acid were lower at puberty in heifers fed maize silage based diets, compared to heifers fed lucerne silage both with higher rates of body weight gain. Diets or growth rates had no effect on subsequent milk yield. In another study, heifers were fed lucerne silage or maize silage plus soybean meal for daily body weight gains of 725 or 950 g during the pre-pubertal stage. Feed intake, milk production and milk composition during first lactation were not affected by the experimental diet or growth rates (Waldo et al., 1998). Carson et al. (2004) fed heifers a straw/concentrate based diet during winter periods had beneficial effects on mammary parenchyma development compared with those on grass silage based diets and pasture grazing during summer improved mammary development over maintaining animals indoors on a straw/concentrate based diet.

The effect of accelerated growth diets during pre-pubertal phase on the early onset of puberty is now widely accepted, but the effects on mammary development are equivocal and impacts on subsequent milk yield are not clearly defined. The majority of the studies showing negative effects of pre-pubertal accelerated dietary on the first lactation are confounded with the management practices, breeding and pregnancy because of differences regarding age and body weight at first calving.

The negative effect of high feeding level on first lactation milk yield was similar among breeds and start when feed rate results in body weight gains above 350, 550 and 650 g/day in Jerseys, Danish Reds and Danish Friesians, respectively (Hohenboken et al., 1995). There are a number of experiments in which the negative effect of feeding level on subsequent milk yield was not observed (Van Amburgh and Galton, 1994; Gaynor et al., 1995). This may be

due to very small growth rate differences between treatment groups, a small number of animals or treatment periods outside a critical period (Sejrsen et al., 2000).

Body growth and first lactation milk yield were not different in dairy heifers fed a high forage or high concentrate ration for similar levels of body weight gain before puberty but 150-d milk yield and milk component production were increased (Zanton and Heinrichs (2007)). Similarly, Sejrsen and Foldager (1992) and Carson et al. (2000) showed that first lactation milk yield did not differ between groups when a high concentrate diet was fed in restricted amounts to produce similar body weight gains compared with a high forage control diet. However, Radcliff et al. (2000) found reduced first lactation milk yield when dairy heifers were offered high concentrate diets *ad libitum*.

1.2.6 Impact of Early Nutrition on Later Life

It is known that foetal organs and systems are sensitive to the environment of the uterus and the influence of this environment can lead to long term consequences (Barker, 2004) such as changes in performance and metabolic function of the offspring (Wu et al., 2006). Influence of epigenetic factors for example, DNA methylation and histone modifications are considered an important role in developmental programming of disease (Burdge et al., 2007). DNA methylation provides a useful mechanism for gene silencing, whereas modifications of histone proteins can either allow gene transcription or prevent expression (Jaenisch and Bird, 2003). Environmental challenges in early life, including under or over nutrition, are likely to affect DNA methylation. This is because during the very early phases of embryo development, DNA methylation is extensively reprogrammed.

The concept of metabolic programming was originally established by Barker et al. (1995). The authors conducted epidemiological studies and suggested that maternal undernutrition was highly correlated with an increased risk of health problems such as hypertension, type II

diabetes and cardiovascular diseases of children born experiencing intrauterine growth retardation at adulthood. Low birth weight has been linked in human studies to increased incidence of type II diabetes (Forsen et al., 2000).

It seems that the organs and systems of the body go through sensible periods when they are plastic and responsive to the environment during development. For most organs and systems, the sensible period occurs in utero. Brooks et al. (1995) stated that small women have small babies even in pregnancies after ovum donation when the woman donating the egg is large. Calves born to heifers are smaller than calves born to adult cows.

Nutrition in the earliest stage of infancy is provided solely as milk, in human babies: milk delivered by breast feeding or formula milk from bottle feeding and in calves: milk secreted from mammary gland or using of milk replacer. The evidence to link early life nutrition with disease in later life is overwhelming. In human, a positive effect of breast feeding on cognitive function is widely reported (Evenhouse and Reilly, 2005) and breast feeding appears likely to protect against some immune related diseases later in life for example type-1 diabetes (Eurodiab, 2002) and inflammatory bowel disease (Klement et al., 2004). In dairy cattle, Soberon et al. (2011) evaluated association between milk yield and early life growth and also nutrition. This study showed that for every 1 kg of average daily gain (ADG), heifers produced 1,067 kg more milk during their first lactation.

1.2.7 Heifer Puberty and Fertility

Age at puberty in heifers is an important predictor of lifetime productivity in both dairy and beef cattle production systems (Patterson et al., 1992; Serjsen and Purup, 1997). For dairy herds it should occur by 12 months of age. Taylor (2001) found a range in ages of 7.5 to 13.5 months for Holstein Friesian heifers with an average of 9.5 months using plasma

progesterone measurements to confirm ovulation and first luteal function. Both, body weight and age are the major influences on the timing of puberty (Mourits et al., 1997).

The circulating concentration of IGF-1 is highly correlated to body weight and growth rate during the period of pre-pubertal growth (Lammers et al., 1999; Brickell et al., 2009). Insulin, concentrations, which generally reflect energy status and dietary adequacy, may be a primary link between the metabolic and reproductive systems. Insulin is necessary to increase synthesis of IGF-1 in the liver in response to elevated concentrations of somatotropin (growth hormone). IGF-1 increases oestradiol production by the dominant follicle and increase LH receptors on the follicle required for ovulation and corpus luteum (CL) development (Lucy, 2000; Garnsworthy et al., 2008).

Poorly grown heifers require more services per conception, calve later and are likely to be culled early (Wathes et al., 2008), thus good growth rates are essential. Heifers achieve puberty at more than 40% of their mature weight and should reach 55-60% of their mature weight before service, about 375-420 kg or more (Wood and Barrett, 2014). They should then calve at 550-600 kg or more with a body condition score of 2.50-3.00 for the Holstein breed (Wood and Barrett, 2014). To achieve this, growth rates of 0.7 kg/day before puberty and 0.8 kg/day after puberty are the minimum requirement and growth of 100 g/day above these minimum targets may be desirable in larger Holstein (Wood and Barrett, 2014). It is essential that growth of heifer is monitored to allow individual animals that are failing to be identified. Measurement of withers height is also important as it measures the frame of the heifer, because tall and lean heifers are more likely to be productive than short and fat heifers (Zanton and Heinrichs, 2006).

The decision on when to start breeding is based primarily on the age of the heifer but is also influenced by growth during the rearing period. In order to achieve an age at first calving (AFC) of 23 to 25 months (Wathes et al., 2008; Cooke and Wathes, 2014), breeding needs to

start at around 13.5 months. This is to minimise costs for rearing and more importantly the future performance of heifers that calve at 23 to 25 months of age is better. They are more likely to survive longer in the herd, with more days in milk and higher lifetime milk yield. In the UK, AFC is on average 27 months (Brickell et al., 2009).

Rapid progress in genetics and management in the dairy industry has resulted in increased milk production per cow. Metabolic demands for more milk production negatively impact the reproductive function of postpartum cows (Beam and Butler, 1999). Rapid increase in energy requirements at the onset of lactation results in negative energy balance (NEB) that begins a few days before calving and usually reaches its most negative level about 2 weeks later (Butler and Smith, 1989; Bell, 1995). From several studies, NEB during the first 3 weeks of lactation is highly correlated with the interval to first ovulation. Cows in high body condition score at calving exhibit decreased appetite, develop more severe NEB undergo increased mobilisation of body fat and accumulate more triacylglycerols in the liver (Rukkwamsuk et al., 1999) which are associated with a longer interval from calving to first ovulation and reduced fertility compared to cows of moderate condition (Garnsworthy and Topps, 1982; Butler and Smith, 1989; Rukkwamsuk et al., 1999).

There are many factors that can influence the function of the ovaries of postpartum dairy cattle. The resumption of ovarian activity plays an important role in their subsequent fertility (Darwash et al., 1997). A wave of follicular development occurs in 5-7 days postpartum regardless of NEB and in response to an elevation in plasma FSH concentrations as the suppression of FSH due to elevated oestrogen concentrations at the end of gestation is removed. The initiation of a follicular wave and formation of a large dominant follicle during NEB does not appear to be a limitation for first ovulation. However, Beam and Butler (1997) described 3 possible outcomes of follicular development: (i) ovulation of the first dominant follicle (16-20 days postpartum); (ii) non-ovulation of the first dominant follicle followed by

turnover and a new follicular wave and (iii) the dominant follicle fails to ovulate and becomes cystic. The development of non-ovulatory dominant or cystic follicles prolongs the interval to first ovulation to 40 or 50 days postpartum. The frequency of LH pulses is significantly lower during the first follicular wave postpartum in cows that fail to ovulate their first dominant follicle compared to those that do Beam and Butler (1999).

First ovulation in both dairy and beef cows generally is not accompanied by behavioural oestrus (Kyle et al., 1992) and is followed by a short cycle, usually containing just one follicle wave. The first luteal phase is shortened in length because of the premature release of prostaglandin F_{2α} (Peter et al., 1989). Corpus luteum regresses prematurely at days 8-10 of the cycle, with the second ovulation (post-ovulatory dominant follicle) occurring at days 9-11 after the first ovulation. The second ovulation is generally associated with the expression of oestrus and a normal length of subsequent luteal phase.

Although cows can have two, three or four follicle waves during the oestrous cycles that occur in the postpartum period (Savio et al., 1990; Sartori et al., 2004). Holstein cows tend to have two follicle waves per 18-23 days cycle (Sartori et al., 2004). Progesterone concentration is the major factor that affects LH pulse frequency in cyclic cows. Generally, cows with prolonged luteal phases tend to have a fourth follicle wave (Savio et al., 1990). Follicle waves also continue during early pregnancy (Savio et al., 1990).

1.2.8 Classification of Progesterone Profiles

The progesterone profiles of the individual cows have been categorised into normal and 4 different patterns of abnormal ovarian activity (Bulman and Wood, 1980; Lamming and Darwash, 1998). Normal cycles have milk progesterone concentrations of >3 ng/ml within 45 days postpartum and regular cycles thereafter, with luteal phases of up to 19 days and inter

luteal phases of less than 12 days. Abnormal categories are considered as 2 types of either delayed ovulation (DOV) or persistent corpus luteum (PCL) depending on when they occurred during postpartum.

- (i). DOV type 1 (DOV1) occurred in the immediate post-calving period, where progesterone concentrations are <3 ng/ml for at least 45 days postpartum.
- (ii). DOV type 2 (DOV2) occurred after cyclicity has resumed post-calving with progesterone concentrations <3 ng/ml for greater than 12 days between adjacent luteal phases.
- (iii). PCL type 1 (PCL1) occurred in the immediate post-calving period, where progesterone concentrations are >3 ng/ml for at least 19 days during the first cycle postpartum.
- (iv). PCL type 2 (PCL2) occurred after the first (normal) cycle post-calving, where progesterone concentrations are >3 ng/ml for at least 19 days during the second or subsequent cycles.

1.2.9 Negative Energy Balance (NEB)

It is well known that NEB is a problem of early lactation cows arising from high milk energy output and relatively low feed intake. However, NEB is also a problem for late gestation cows (Grummer et al., 2004) and may predispose them to many transition cow disorders for instance dystocia (Zamet et al., 1979), retained placenta (Cameron et al., 1998), fatty liver and ketosis (Doherty, 2002), reduced feed intake after calving (Doepel et al., 2002), immune-suppression (Goff, 2003) and displaced abomasum (LeBlanc et al., 2005). Transition period can be defined as 3 weeks before until 3 weeks after calving (Grummer, 1995), is period during which the cows experience severe NEB due to decreased dry matter intake and increased energy demand for lactation. The stress of the transition period can result in severe lipid mobilisation and an excessive elevation of circulating ketone bodies (Herdt, 2000) and non-esterified fatty acid (NEFA). Ketone bodies (β -hydroxybutyrate, acetone and

acetoacetate) are intermediate metabolites of fatty acid oxidation. Subclinical ketosis (SCK) and clinical ketosis (CK) result in increased concentrations of ketone bodies in tissues and milk (Enjalbert et al., 2001). Several blood metabolites have been used to monitor metabolic changes during the transition period and to predict the risk of postpartum disorders (LeBlanc et al., 2005). The concentrations of BHB, NEFA and glucose have been used to assess the adaptive response to energy balance (Wathes et al., 2007; Jackson et al., 2011). Cows with SCK, can be defined as blood β -hydroxybutyrate (BHB) of 1.2 to 2.9 mmol/L and values ≥ 3.0 mmol/L indicate CK (Oetzel, 2004; Duffield et al., 2009). Compared with non-ketotic cows, the cows with ketosis had significantly higher level of BHB and NEFA and lower levels of glucose in early lactation (Asl et al., 2011). Other blood metabolites for instance aspartate aminotransferase (AST) and γ -glutamyltransferase (γ GT) might be useful in monitoring the fatty liver that commonly occurs at parturition (Bobe et al., 2004).

During lactation, dairy cows have very high nutritional requirements relative to most other species. Meeting these requirements, especially for energy and protein is challenging. Diets must have sufficient nutrient concentrations to support production and metabolic health, while also supporting rumen health and the efficiency of fermentative digestion.

Carbohydrates (mainly fibre, starch and soluble sugars) and protein in the diet provide substrates for rumen fermentation which results in the production of volatile fatty acids (VFA). The main VFA produced are acetate, propionate and butyrate. Acetate and butyrate split into fragments containing two carbon atoms (C2) and are considered lipogenic nutrients.

Propionate is a fragment containing three carbon atoms (C3) and is considered a glucogenic nutrient. Dietary ingredients that are resistant to rumen degradation can be digested and absorbed in the intestine and provide either lipogenic or glucogenic nutrients. The final common pathway for oxidation involves the oxidation of a lipogenic nutrient (acetyl-

coenzyme-A) and a glucogenic nutrient (oxaloacetate) to form citrate. Citrate proceeds through a series of intermediate reactions in the Krebs cycle and respiratory chain reaction to make energy available for the body as adenosine triphosphate (ATP). This implies that providing acetyl-coenzyme-A and oxaloacetate in a one to one ration is ideal for efficient metabolism and generation of energy to the body.

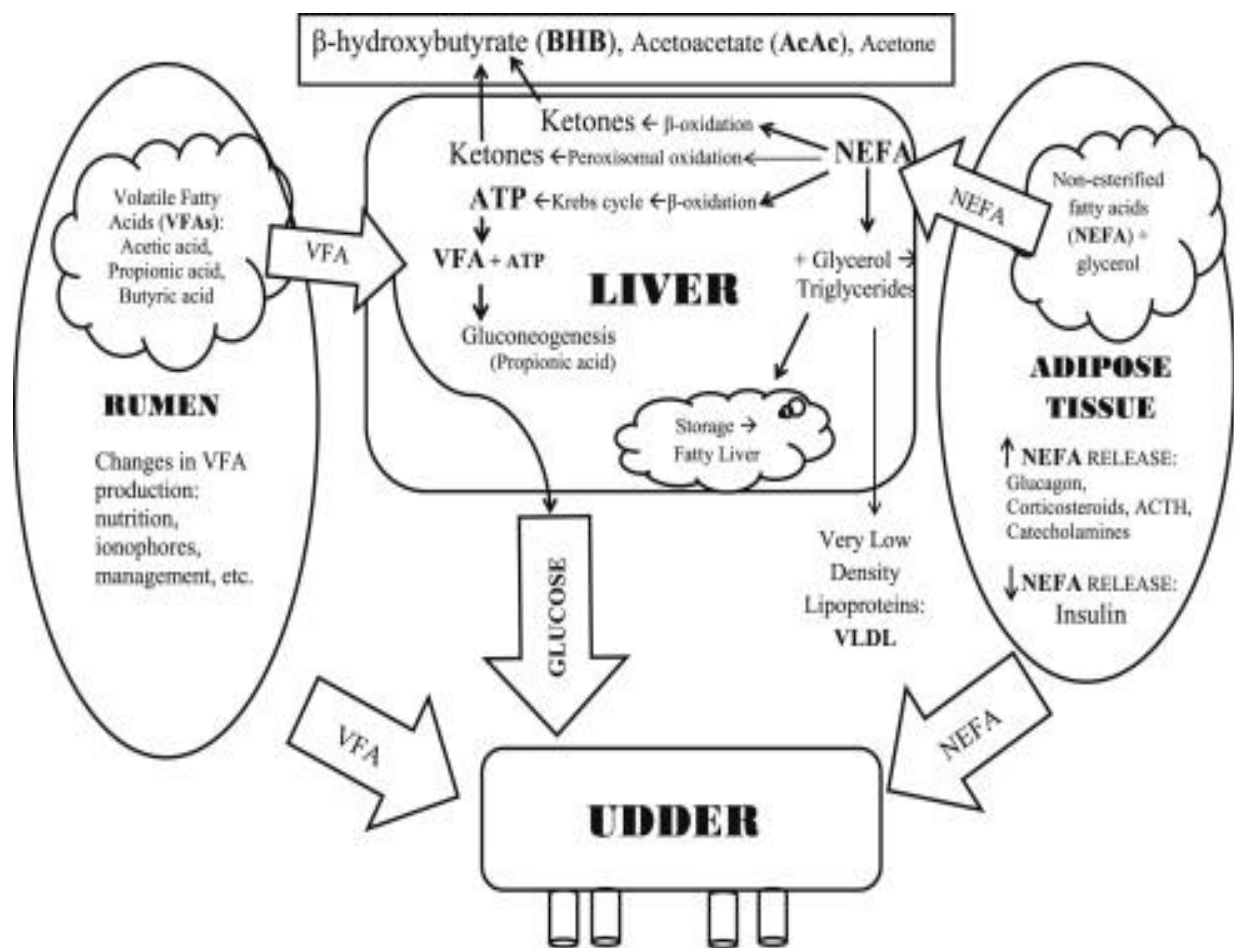


Figure 1.1 The relationship between energy demands, energy reserves and the metabolic association between non-esterified fatty acids (NEFA) and β -hydroxybutyrate (BHB); from Ospina et al., 2013. Abbreviations: ACTH, corticotropin; ATP, adenosine triphosphate.

In early lactation energy intake is usually lower than energy requirements for maintenance and milk production, which results in negative energy balance and mobilisation of body reserves. Figure 1.1 - illustrates the energy metabolism of lactating dairy cows during NEB. Body reserves are mainly body fat, which is a source of lipogenic nutrients. Mobilisation of body fat results in elevated plasma non-esterified fatty acid (NEFA) concentration, which can be oxidized to acetyl-coenzyme-A. High milk production in early lactation requires high lactose production from glucogenic nutrients which is facilitated by low plasma insulin concentration. In cows during NEB, the availability of lipogenic nutrients for the production of acetyl-coenzyme-A is increased whilst glucogenic nutrients are driven towards lactose. Consequently, the ratio of oxaloacetate to acetyl-coenzyme-A is out of balance and the production of citrate to form ATP is limited. Alternatively, acetyl-coenzyme-A is diverted to the production of ketones, acetone, acetoacetate and β -hydroxybutyrate (BHB), eventually resulting in a status of ketosis. An excess of lipogenic nutrients result in esterification and storage of fatty acids as triacylglycerides (TAG) in the liver, which may result in fatty liver. Fatty acids can be esterified and transported as very-low-density-lipoproteins (VLDL) to the mammary gland resulting in an increased milk fat production. However, the extent of VLDL transport in cows is limited.

Dairy cows with metabolic and reproductive disorders in early lactation may suffer from an imbalance in availability of C2 and C3 compounds induced by the NEB. However, the availability of C2 and C3 compounds can also be manipulated by adapting the dietary ingredients in the cow's ration. Lipogenic nutrients are expected to increase the C2-C3 compound ratio, while glucogenic nutrients decrease the C2-C3 compounds ratio.

High milk producing dairy cows experience a substantial increase in energy requirements to facilitate the dramatic increases in daily milk yield, which peaks between 4 and 8 weeks postpartum. This requirement is only partially met by increased feed consumption (due to

limitations in intake and appetite) with the remainder being met by mobilisation of body reserves resulting in cows entering negative energy balance (Grummer, 2007). The consequences of severe NEB are an increased risk of metabolic diseases, which largely occur within the first month of lactation, a reduction in subsequent fertility and reduced immune function (Roche et al., 2009).

On the other hand, there are 3 basic strategies to minimise the duration of NEB and BCS loss in early lactation. The first strategy is to reduce BCS at calving so that energy intake is not limited by the negative feedback effect of BCS. The second strategy is to feed low protein diets that reduce body fat mobilisation (Garnsworthy and Jones, 1987; Westwood et al., 2000). The third strategy is to increase dietary energy concentration by increasing the starch or fat components of the diet at the expense of forage components (Garnsworthy et al., 2008).

1.2.10 Body Condition Scoring (BCS) as an Indirect Predictor of Fertility

Body condition scoring provides a rapid, simple and acceptably precise estimate of body fatness, as shown in Appendix A Table A.1 (Edmonson et al., 1989; Ferguson et al., 1994). It is an internationally accepted, subjective visual and tactile measure of body condition and temporal changes in BCS are used to monitor nutritional and health status of high producing cows during their productive cycle (Berry et al., 2007). It has been correlated with reproductive performance, both phenotypically (Buckley et al., 2003) and genetically (Berry et al., 2003) and supports the premise that nutritional status affects reproductive function.

Cows in low BCS at calving, or that suffer excess BCS loss early postpartum, are less likely to ovulate, have a reduced submission rate to artificial insemination, conception rate to first service, have an increased likelihood for pregnancy loss and increased calving to conception

interval (Berry et al., 2007; Roche et al., 2009). This can partly be attributed to impaired oocyte competence associated with a low BCS (1.50-2.50; 5-point scale) (Snijders et al., 2000). Fertility in cows that are over conditioned at calving ($BCS \geq 3.50$; 5-point scale) is also compromised as they have reduced dry matter intake (DMI) just prior to calving, take longer to increase DMI postpartum, tend to have greater fat mobilisation and therefore a more severe NEB early postpartum than cows with an optimum BCS at calving (Roche et al., 2009).

Numerous studies have examined the association of BCS with health and reproduction, and while generally cows that calve in fat body condition, or moreover cows that lose 1 point or more of BCS in early lactation, are often reported to be at higher risk of adverse outcomes. BCS alone (other than extremes i.e. >4.00 or <2.50 at calving) is not a sensitive or specific tool for prediction of disease or reproductive performance. Recent research has suggested that the target BCS at calving should likely be lower (≤ 3.00) than previously advocated to optimize health and production (Garnsworthy, 2008). It is recommended that cows have a BCS of 2.75-3.00 (scale 0-5) at calving and that they are managed to suffer a BCS loss not more than 0.5 between calving and first service (Crowe, 2008). Several studies reported that a BCS of 3.50 or greater at calving was associated with increased risk for ketosis (Duffield, 2000; Gillund et al., 2001). Fat cows lose more body condition during early postpartum than do thin cows (Kim and Suh, 2003). Cows with high BCS at calving tended to limit feed intake during postpartum, which predisposed them to severe postpartum body condition loss (Rukkwamsuk et al., 1999; Wathes et al., 2007).

1.2.11 Rumen Fill Score as a Predictor of Rumen Health

Rumen fill was estimated by visual evaluation of the paralumbar fossa on a scale from 1 to 5 (Appendix A Table A.2 – Rumen fill score and appearance of paralumbar fossa) according to

the system of Zaaijer and Noordhuizen (2003). It can be defined as the total amount of liquid and dry matter (kg) in the rumen, related to dry matter intake (Hartnell and Satter, 1979), ration composition, digestibility and the rate of passage of ingested feed (Llamas-Lamas and Combs, 1991). Visual examination is an important component in health evaluation of cows, particularly in early lactation (Guterbock, 2004; Smith and Risco, 2005).

1.2.12 BHB and NEFA as Predictor of Disease, Production and Reproduction

Subclinical ketosis (BHB > 1200 to 1400 $\mu\text{mol/l}$ depending on study) in the first or second week after calving is associated with: 3 to 8 times increased risk of displaced abomasum (DA) (LeBlanc et al., 2005; Duffield et al., 2009); 3 times greater risk of metritis when serum BHB in week 1 was > 1200 $\mu\text{mol/l}$ (Duffield et al., 2009); 4 to 6 times increased risk of clinical ketosis (Duffield et al., 2009) and increased duration and severity of mastitis (Suriyasathaporn et al., 2000) however, not with the incidence of mastitis (Duffield et al., 2009). Milk production at first test was reduced by 1.9 kg/d when BHB was > 1400 $\mu\text{mol/l}$ in week 1 and by 3.3 kg/d when BHB was > 2000 $\mu\text{mol/l}$ in week 2. However, cows with serum BHB > 1800 $\mu\text{mol/l}$ in week 1 had > 300 kg lower projected milk yield for the whole lactation (LeBlanc et al., 2005).

Ketosis is associated with reduced reproductive performance, which extends its impact much longer than many farmers realise. It is worth emphasizing that health in the weeks before and after calving influences reproduction at least 2 months later. For example, cows with BHB > 100 $\mu\text{mol/l}$ in milk in the first week postpartum were 1.5 times more likely to be anovular at 9 weeks postpartum (Walsh et al., 2006). Besides that, cows that experienced ketosis in the first two weeks of lactation had reduced probability of pregnancy at the first insemination. Furthermore, cows that had ketosis in one or both of the first two weeks after calving had a lower pregnancy rate until 140 DIM.

High NEFA (>0.4 mmol/l) in the last 7 to 10 days before expected calving is associated with: 2 to 4 times increased risk of DA (LeBlanc et al., 2005); 2 times increased risk of retained placenta (LeBlanc et al., 2004); 2 times increased of culling before 60 days in milk (DIM) and 1.5 times increased risk of culling over the whole lactation (Duffield et al., 2005).

1.2.13 Metabolites to Measure Energy Status in Transition Cows

Circulating concentrations of BHB and NEFA measure aspects of the success of adaptation to negative energy balance. The concentration of NEFA reflects the magnitude of mobilisation of fat from storage and mirrors dry matter intake (DMI) (Adewuyi et al., 2005); while BHB reflects the completeness of oxidation of fat in the liver. Ketone bodies (BHB, acetone and acetoacetate) are the intermediate metabolites of oxidation of fatty acids, specifically resulting from the incomplete oxidation of fatty acids to acetyl CoA. As the supply of NEFA to the liver exceeds the ability of liver to completely oxidise the fatty acids to supply energy, the amount of ketone production increases. Increasing concentrations of ketone are thought to suppress feed intake (Allen et al., 2009).

Blood urea is the metabolic end product of protein catabolism in the body and is thought to reflect the dietary balance between effective rumen-degradable protein (eRDP) and fermentable metabolisable energy (Butler, 1998).

Glucose is the primary metabolic fuel and is required for vital organ function, foetal growth and milk production. In dairy cows, the massive energy demand to support milk production is largely met through gluconeogenesis. Glucose concentrations are under tight homeostatic control. Although glucose has a main role in metabolism, it is a poor analyte for monitoring or investigating herd problems (Herdt, 2000).

1.2.14 Milkbot Lactation Model

MilkBot (MB) model was developed by Ehrlich (2011). It is a nonlinear lactation model which provides a means of quantifying both shape and magnitude of lactation curves as a set of parameter values. Lactation data may be fitted to the model to summarise a lactation as a set of parameter values which summarise the lactation as a whole. The *scale* parameter controls magnitude without changing the shape of the curve; the *ramp* parameter controls steepness of the post-parturient rise in milk production; the *decay* parameter controls the rate of late lactation decline and the *offset* parameter defines a theoretical offset between the start of milk production and calving. The *decay* parameter is easily re-expressed mathematically as *persistence* to quantify the rate of decline in production after peak milk. Time and quantity of peak milk or production for any day or period in the lactation may be calculated directly from parameter values.

Many lactation models have been proposed and compared over the past half century (Wood, 1967; Rook et al., 1993; Norman et al., 1999; Grossman et al., 1999); however, none have achieved widespread acceptance outside a few specialised applications which are directed primarily at improving estimates of actual production from incomplete data sets. MB model has close mathematical similarities to some of these earlier models, for example Mitscherlich-Exponential model proposed by Rook et al. (1993) but differs in that the model is derived from a theoretical mechanistic hypothesis (Ehrlich, 2006), leading to parameters which can be interpreted both in terms of the effect that they have on the curve and in terms of the mechanistic hypothesis. This is importance to the interpretability of fitted parameter values which become metrics in their own right of the distribution of milk production within a lactation or lactation curve shape. The term ‘persistency’ has been used to quantify the rate of decline in milk production in the later part of lactation. But definition of persistency vary widely (Grossman and Koops, 2003; Cole and VanRaden, 2006) and there is no standard

definition. Similarly 'peak milk', meaning the highest daily production; while strictly a measure of magnitude, can be used to quantify shape when compared to cumulative production. It can be observed that some lactations rise more steeply after calving than others. An attempt to quantify this aspect of curve shape is 'time to peak milk' which has the disadvantage of being dependent on frequency of data collection. Difficulties in calculation and accuracy of these measures, with lack of clear definitions in some cases have kept them from wide application.

Chapter 2

General Materials and Methods

General Materials and Methods

This chapter describes materials and methods which are common to subsequent chapters. Specific materials and methods are detailed in the relevant chapter.

2.1 *Animals, Study Design and Feeding*

The present study was conducted from 4th April 2013 until 16th April 2015 on 88 pedigree Holstein heifers at Wood Park Farm, University of Liverpool, United Kingdom. Heifers were between 23-32 months of age. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 under a UK Home Office licence for work on living animals and with the approval of the University of Liverpool Ethical Review Process.

Heifers in Group A - *ad libitum* (forty seven animals) and Group R - restricted (forty one animals), were enrolled 8 weeks prior to predicted calving based on artificial insemination dates of pregnancies confirmed by per rectum ultrasound examination of the uterus and 282 days of gestation period. Briefly, heifers in Group A (*ad libitum*) had free access to milk replacer as calves teat fed from automatic feeder, were group-housed from birth and weaned at 12 weeks old. Heifers in Group R (restricted) were fed 3 litres of milk replacer twice daily via a bucket or trough, housed individually for the first 3 weeks of life and then group housed until weaning at 8 weeks old (Gemma, 2015).

During this study, heifers/cows were kept inside and fed a Total Mixed Ration (TMR) *ad libitum* formulated by DIETPLAN DAIRY PRO computer programme (W. Morris, ForFarmers PLC, Ipswich, UK). Two months before predicted calving date, heifers were group housed in a straw yard with access at a feed barrier to a transition dry cow TMR that contained 48.4% dry matter, 14.0% DM crude protein, 4.96% DM sugar, 4.8% DM starch

and 50.4% DM NDF with an overall energy density (M/D) of 9.8 MJ ME/kg DM. Average DM intake in the group was estimated to be 12 kg daily. After calving, heifers were housed in an adjacent straw yard until 21 days and then moved to cubicle housing with milking cows. They were fed an identical high yielder TMR ration at a feed barrier in both locations that contained 49.6% dry matter, 16.8% DM crude protein, 6.0% DM sugar, 21.7% DM starch and 32.8% DM NDF with an overall energy density (M/D) was 12.2 MJ ME/kg DM. Estimated DM intake was 25.0 kg daily (including all the cows regardless of lactation). Animals were fed with a low yielder TMR ration 10 weeks before they were dried-off that contained 48.7% dry matter, 16.2% DM crude protein, 6.3% DM sugar, 19.8% DM starch and 36.2% DM NDF with an overall energy density (M/D) of 11.5 MJ ME/kg DM. Estimated group DM intake was 20.8 kg daily.

2.2 Physical Measurements

2.2.1 Body Weight and Measurements

The following measurements were taken for each animal 8, 4, 2 and 1 week prior to predicted calving, on the day of calving and at week 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 20 and 30 of postpartum by the author. Weighed using Tru-Test® electronic weighing equipment (Ritchey Ltd., North Yorkshire, UK), heart girth, belly girth, crown rump length and hock fetlock length using a plasticised tape measure, height at wither and height at loins using a wooden measuring stick (I and D Smallwood, UK), body condition score using the visual technique developed by Edmondson et al. (1989) and rumen fill by visual evaluation of the paralumbar fossa on a scale from 1 to 5 according to the system developed by Zaaijer and Noordhuizen (2003).

At birth, all calves born alive were weighed using Tru-Test® electronic weighing equipment (Ritchey Ltd., North Yorkshire). The following measurements were also taken at this time: heart girth, belly girth, height at withers and at loins, crown rump length and hock fetlock length using the similar equipment as heifers by the author.

2.3 Samples

2.3.1 Blood Sampling

Blood samples were collected by coccygeal/jugular venipuncture into plain and heparinised 10 ml vacutainers (Beckton Dickinson & Son Ltd., Oxford, UK) from all heifers started from 8, 4, 2 and 1 week before expected date of calving, on the day of calving and at week 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 20 and 30 of postpartum by the author. All heifers were tested for β -hydroxybutyrate (BHB) concentration measurements using a portable BHB meter (Abbott Laboratories Ltd., UK).

2.3.2 Milk Sampling

Milk samples for hormone analysis were collected into 7 ml Bijou container (Scientific Laboratory Supplies Ltd., UK) twice weekly (Monday and Thursday) during afternoon milking by farm staff then checked and frozen at -18°C without preservative by the author. Sampling began from first week of postpartum until 10 weeks of postpartum.

2.4 Milk Yield

Animals were machine-milked 2 times daily during the transition period (first 3 weeks after calving) and then 3 times daily. Individual cow milk yield was recorded daily using commercial software (GEA DairyPlan C21 Version 5.2, GEA WestfaliaSurge, United Milking Systems Ltd., UK). Individual cow milk samples were collected monthly and analysed at the Cattle Information Service (CIS), Rickmansworth, UK, for fat percentage, protein percentage and somatic cell counts (SCC).

Chapter 3

Effect of Different Pre-Weaning Feeding Strategies in Early Life of Heifer Calves on Growth and Day Old Calf Performances

3.1 Introduction

The main goal of the dairy farmers is to produce the best heifers from their replacement heifers. Rearing replacement dairy heifers is an important task to dairy production. They are grown with the objective that these female animals eventually enter the milking herd and produce milk according to their genetic potential. Replacement dairy heifers are generally raised on their farm. However, replacement heifer management and feeding is expensive and requires extensive labour. It costs about 20% of dairy farm expenses (Heinrichs, 1993).

Growth can be defined as maturation of the reproductive system, as well as an increase in body size and weight and is affected by many factors such as genetics, nutrition and management (Heinrichs and Hargrove, 1987). Swanson (1967) defined the optimal growth pattern for dairy heifers as a regimen that will develop their full lactation potential at the desired age and with minimum expense.

A number of factors that include body weight, body size, nutrition, breed, season and social environment influence age at the onset of puberty (Moran et al., 1991). Reducing the age of puberty onset allows early breeding and therefore, reduces the age at first calving (AFC), minimising the expense of dairy heifers rearing. A study conducted by Heinrichs et al. (2005) reported that age at first calving was affected by nutritional, health, events around birth and environmental factors imposed during the first 4 months of life. To achieve this, high concentrate diets that may have high energy and high protein are being fed to accelerate growth rates of calves and heifers. Bortone et al. (1994) reported that heifers fed 115% of National Research Council (NRC) recommendations from 3 to 12 months of age were 22 days younger at the onset of puberty than control animals fed 100% of NRC levels. An increased growth rate in heifers reduces the age at puberty (Schillo et al., 1992; Heinrichs, 1993).

Body condition score (BCS) is an easy and inexpensive method to evaluate the body tissue reserves of lactating cows independently of the frame size and body weight (Edmonson et al., 1989). During early lactation period, the mobilisation of body reserves for milk production induces a negative energy balance that has been reported to affect the reproductive performance of dairy cows (Beam and Butler, 1999; Gillund et al., 2001; Buckley et al., 2003). Therefore, many factors for example feeding system and level, system of milk production, cow's genetic background and parity might influence the results of studies.

Dairy calves are among the few farm animals that are subjected to restricted milk intake in early life. It has been proposed that increased feeding amount of milk replacer during the pre-weaning phase can lead to improvements in heifer performance in later life (Van Amburgh et al., 2001). However, there is very little scientific evidence for these longer term benefits. Several studies have investigated the effects of increasing the supply of milk to young calves (Diaz et al., 2001; Jasper & Weary, 2002; Brown et al., 2005; Khan et al., 2007). These studies have reported higher weight gains and more natural behaviour in calves were observed when the calves fed more milk. The disadvantages of providing more milk are reduced solid feed intake during the milk feeding period (Terre et al., 2007) and slower rumen development (Khan et al., 2007).

Excessive starch feeding can predispose the animal to ruminal acidosis and laminitis (Lean et al., 2013). This disorder can affect performance of the animal and will reduce the rate of gain.

Nutrition is a main aspect of determinant for immune responses, with the protein and energy supply influencing cell mediated immunity, cytokine production, complement system, phagocytic functions and secretory IgA antibody concentrations (Galyean et al., 1999). Smith et al. (2002) reported that increased nutrient intake sufficient to optimise growth performance was associated with elevated plasma IGF-1, insulin and glucose concentrations in calves.

Their study also found that in well-nourished calves, the somatotrophic axis is functionally coordinated with nutrient intake and growth hormone but not in undernourished calves. Nutritional deficiency can suppress immune function and thus increase susceptibility to diseases in calves (Nonnecke et al., 2003).

Health and management systems of dairy cattle should be focused on early identification and prevention of production diseases such as clinical and subclinical ketoses. Clinical and subclinical ketoses are important metabolic diseases in dairy cattle during early lactation and are associated with losses in milk production and other periparturient diseases. Ketosis is a disease related to the high rate of glucose utilisation in the mammary gland and the inability of some cows to meet this glucose demand through normal physiology (Baird, 1982). Subclinical ketosis (SCK) is defined as elevated concentrations of circulating ketone bodies in the absence of clinical signs of ketosis (Andersson, 1988). It is a common disease in high production dairy cows and is caused by a negative energy balance which can affect milk yield (Dohoo and Martin, 1984) and reproduction (Andersson, 1988; Whitaker et al., 1993). Ketone bodies include β -hydroxybutyrate (BHB), acetoacetate and acetone. BHB is synthesised from absorbed butyrate in the ruminant epithelium and by ketogenesis in hepatocytes as they convert long chain fatty acids during fat mobilisation. Both acetoacetate and BHB are freely distributed and transported in the blood and interconvertible in various tissues (Bruss, 1997). It has been shown that SCK can be associated with an increased frequency of left displaced abomasa (Geishauser et al., 1997).

The first objective of the present study was to determine the effect of two different milk replacer feeding strategies in early life of heifer calves, namely *ad libitum* milk replacer feeding (Group A) and restricted milk replacer feeding (Group R); on their future growth in terms of body mass, body condition score, selected morphometric measures and also their day old calves performances.

The second objective of the present study was to evaluate and compare the incidence of metabolic disease especially SCK between the 2 groups.

3.2 Materials and Methods

3.2.1 Animals

The present study was conducted from 4th April 2013 until 16th April 2015 on 88 pedigree Holstein heifers at Wood Park Farm, University of Liverpool, United Kingdom. Heifers in Group A – *ad libitum* (forty seven animals) and Group R – restricted (forty one animals). They were between 23-32 months of age. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 under a UK Home Office licence for work on living animals and with the approval of the University of Liverpool Ethical Review Process.

3.2.2 Statistical Analysis

All data were analysed using Stata 13 (StataCorp) with data expressed as mean \pm SEM or proportions where appropriate. They were checked for normality using normal quantile-quantile plots and basic descriptive statistics were carried out. Normally distributed data is described using mean values and standard errors of the mean. However, where data are not normally distributed, medians and inter quartile range (IQR) are quoted.

Data regarding heifer body weight (BW), body condition score (BCS), rumen fill (RF), heart girth (HG), belly girth (BG), crown rump length (CRL), hock fetlock length (HFL), wither height (WH), loin height and β -hydroxybutyrate (BHB) were analysed and compared between the 2 groups using two-sample t-tests. Data regarding calf birth weight, sex, heart girth, belly girth, crown rump length, hock fetlock length, wither height and loin height on the day of calving were compared between the 2 groups using two-sample t-test. Differences were considered statistically significant if P values were equal or less than 0.05.

Simple correlation coefficients were used to examine the relationships between body measurement and height measurement of the dam (heifer) and body measurement and height measurement of the calf.

Initially, lowess (locally weighted regression) plots were constructed to ascertain the shape of the association between body weight and days in milk (DIM). These suggested a curvilinear association. Both polynomial and log transformed models were considered but fit was poor. Consequently, a fractional polynomial random effects regression model was fitted with postpartum body weight on a specific day postpartum as the outcome variable. Explanatory variables offered to the initial model were heifer associated variables namely pre-weaning dietary group (*ad libitum* milk replacer versus restricted milk replacer), days in milk (DIM), age, BCS, BCS change, BHB concentration, wither height, rumen fill score and milk yield. A backward stepwise method was used for selection of variables for inclusion in the final model taking $P < 0.200$ (likelihood ratio test) for retention of a variable. Interaction terms were offered to the model and retained if they improved model fit as judged by the likelihood ratio test ($P < 0.200$). The variable “pre-weaning dietary group” was forced into the model as the outcome variable of primary interest. Heifer identity was included as a random intercept with DIM as a random slope.

Similarly, a multivariable random effects linear regression model was fitted with postpartum BHB concentration on a specific day postpartum as the outcome variable. Firstly, a lowess plot of BHB against DIM was plotted in order to visualise the shape of the curve. Both polynomial and log transformed models were considered but fit was poor. Thus, a fractional polynomial random effects model was chosen. Explanatory variables offered to the initial model were pre-weaning dietary group (*ad libitum* milk replacer versus restricted milk replacer), age, body weight, BCS, BCS change, rumen fill score, DIM and milk yield. A backward stepwise method was used for selection of variables for inclusion in the final model

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The impact of feeding group (A *versus* R) on the likelihood of an animal developing sub-clinical ketosis (SCK), defined as $\text{BHB} \geq 1.2$ mmol/L (Duffield et al., 1998a), was investigated by fitting a random effects logistic regression model with the outcome variable being $\text{BHB} \geq 1.2$ mmol/L. The following variables were offered pre-weaning dietary group (*ad libitum* milk replacer versus restricted milk replacer), age, body weight, BCS, BCS change, rumen fill score, DIM and milk yield. A backward stepwise method was used for selection of variables for inclusion in the final model taking $P < 0.200$ (likelihood ratio test) for retention of a variable. Interaction terms were offered to the model and retained if they improved model fit as judged by the likelihood ratio test ($P < 0.200$). The variable “pre-weaning dietary group” was forced into the model as the outcome variable of primary interest. Heifer identity was included as a random effect. Frequencies of SCK occurrence between groups were compared with chi-squared test.

Survival analysis was performed to evaluate time for peak BHB concentration. Kaplan-Meier survival curves were plotted for Group A and Group R heifers separately. Survival curves were compared between the 2 groups using the log-rank test (test equality of survivor functions).

Univariable Cox regression models were fitted to investigate the impact of pre-weaning feeding group (A *versus* R) and potential confounders on the time for peak BHB

concentration. Potential confounders considered were: body weight, BW change, BCS, BCS change, rumen fill score and age.

3.3 Results

3.3.1 Heifer Measurement

Descriptive Statistics

Age at First Calving (AFC)

There was no significant difference in AFC between Group A and Group R heifers (Group A: 750.6 ± 10.0 days *versus* Group R: 760.7 ± 12.5 days), ($P=0.529$) (Figure 3.1).

Body Weight on the Day of Calving

There was no significant difference between groups with respect to BW on the day of calving after giving birth (Group A: 566.1 ± 10.3 kg *versus* Group R: 568.7 ± 7.4 kg), ($P=0.836$) (Figure 3.2) and (Figure 3.3).

Body Condition Score on the Day of Calving

There was no significant difference with respect to BCS on the day of calving after giving birth between Group A and Group R heifers (Group A: 2.59 ± 0.03 units *versus* Group R: 2.53 ± 0.03 units), ($P=0.196$) (Figure 3.4).

β -hydroxybutyrate on the Day of Calving

There was no significant difference in BHB concentration on the day of calving after giving birth in Group A and Group R heifers (Group A: 0.48 ± 0.02 mmol/L *versus* Group R: 0.51 ± 0.03 mmol/L), (P=0.495).

Heart Girth on the Day of Calving

There was no significant difference between the 2 groups with respect to HG on the day of calving after giving birth (Group A: 198.7 ± 1.4 cm *versus* Group R: 199.8 ± 1.3 cm), (P=0.569).

Belly Girth (BG) on the Day of Calving

There was no significant difference in BG on the day of calving after giving birth between Group A and Group R heifers (Group A: 238.2 ± 1.6 cm *versus* Group R: 239.6 ± 1.4 cm), (P=0.519).

Wither Height on the Day of Calving

Restricted fed animals tended to be taller at the withers on the day of calving after giving birth (Group A: 143.5 ± 0.6 cm *versus* Group R: 145.1 ± 0.6 cm), (P=0.076) (Figure 3.5).

Loin Height on the Day of Calving

Group R heifers tended to be higher at the loin on the day of calving after giving birth (Group A; 148.4 ± 0.7 cm *versus* Group R: 150.0 ± 0.5 cm), ($P=0.055$).

Crown Rump Length on the Day of Calving

There was no significant difference between the 2 groups with respect to CRL on the day of calving after giving birth (Group A: 220.4 ± 1.7 cm *versus* Group R: 220.9 ± 1.9 cm), ($P=0.838$).

Hock Fetlock Length on the Day of Calving

Restricted fed heifers tended to be longer at the hock fetlock on the day of calving after giving birth (Group A: 55.8 ± 0.4 cm *versus* Group R: 56.6 ± 0.3 cm), ($P=0.065$).

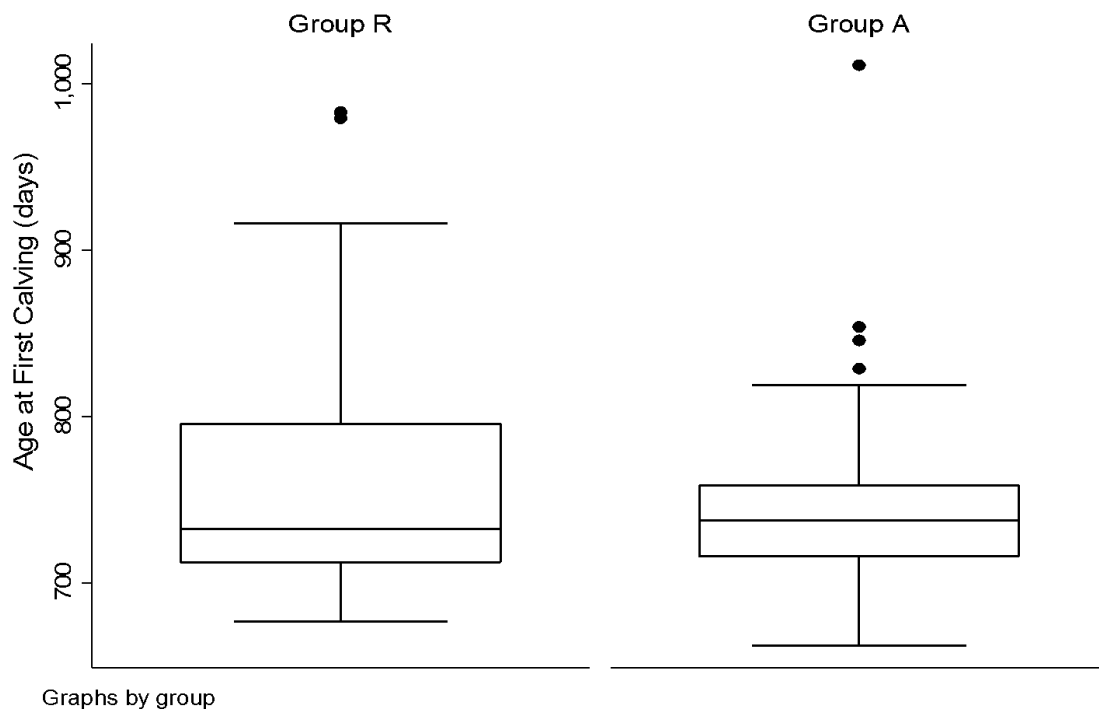


Figure 3.1 Box plot for age at first calving for Group A (*ad libitum*) (n=39) and Group R (restricted) (n=37), (P=0.529).

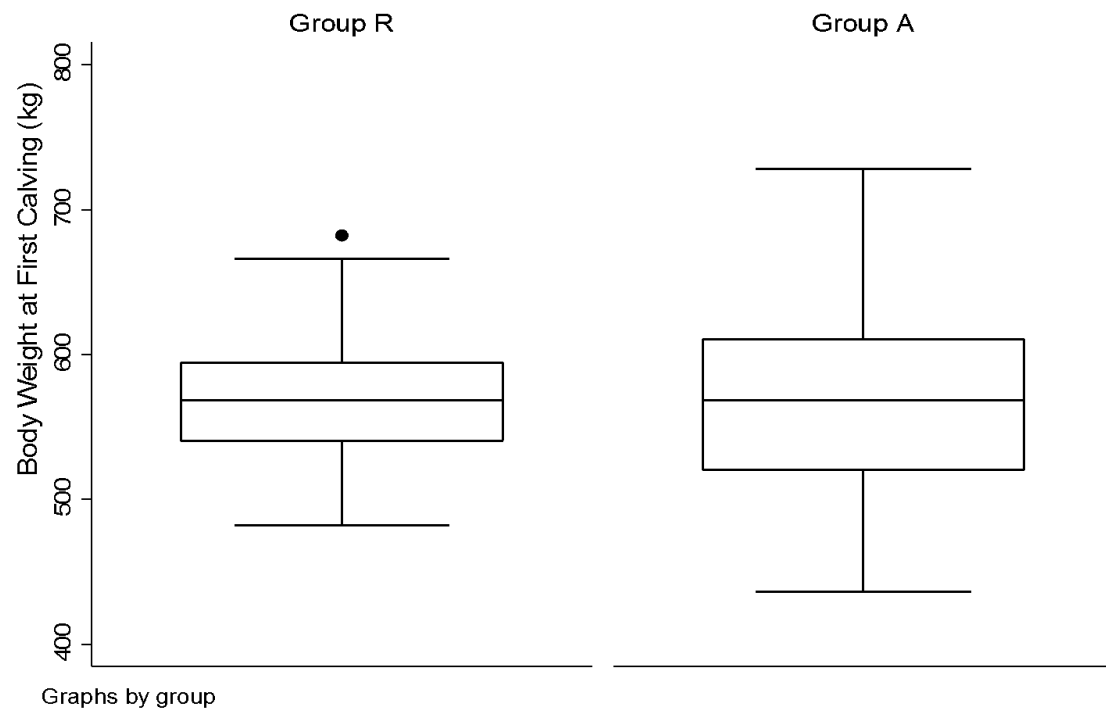


Figure 3.2 Box plot for body weight on the day of calving for Group A (*ad libitum*) (n=39) and Group R (restricted) (n=37), (P=0.836).

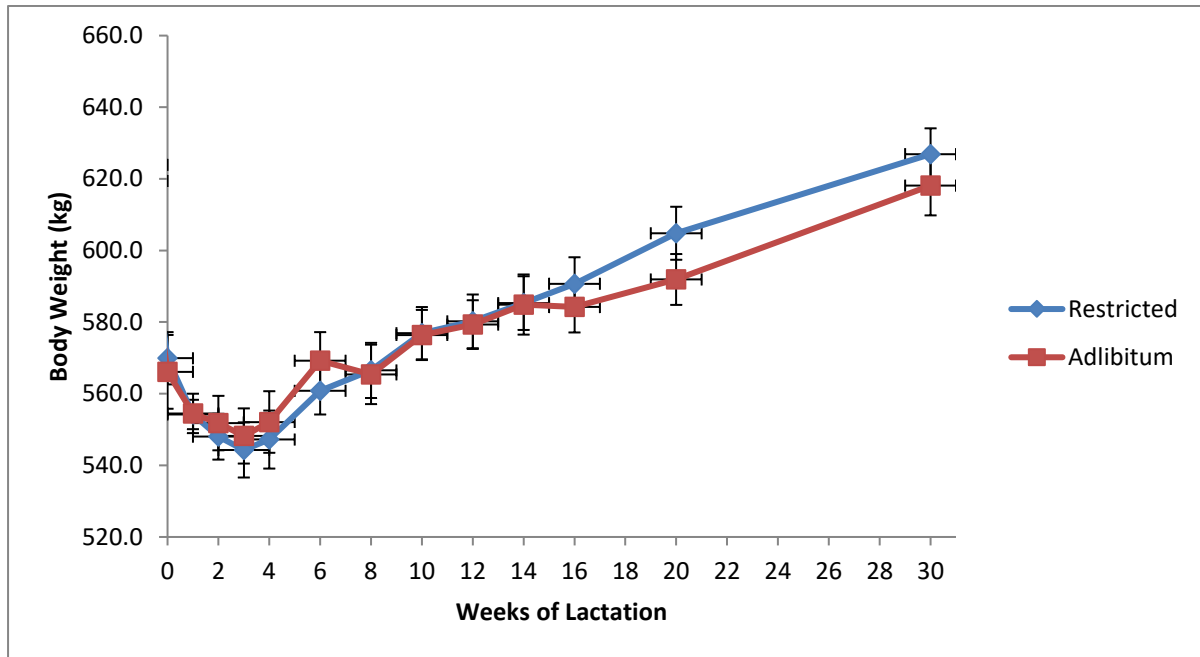


Figure 3.3 Changes in body weight by weeks of lactation for Group A (*ad libitum*) and Group R (restricted). Values presented as mean \pm SEM. Body weight on the day of calving was not different (Group A: 566.1 \pm 10.3 kg *versus* Group R: 568.7 \pm 7.4 kg), (P=0.836).

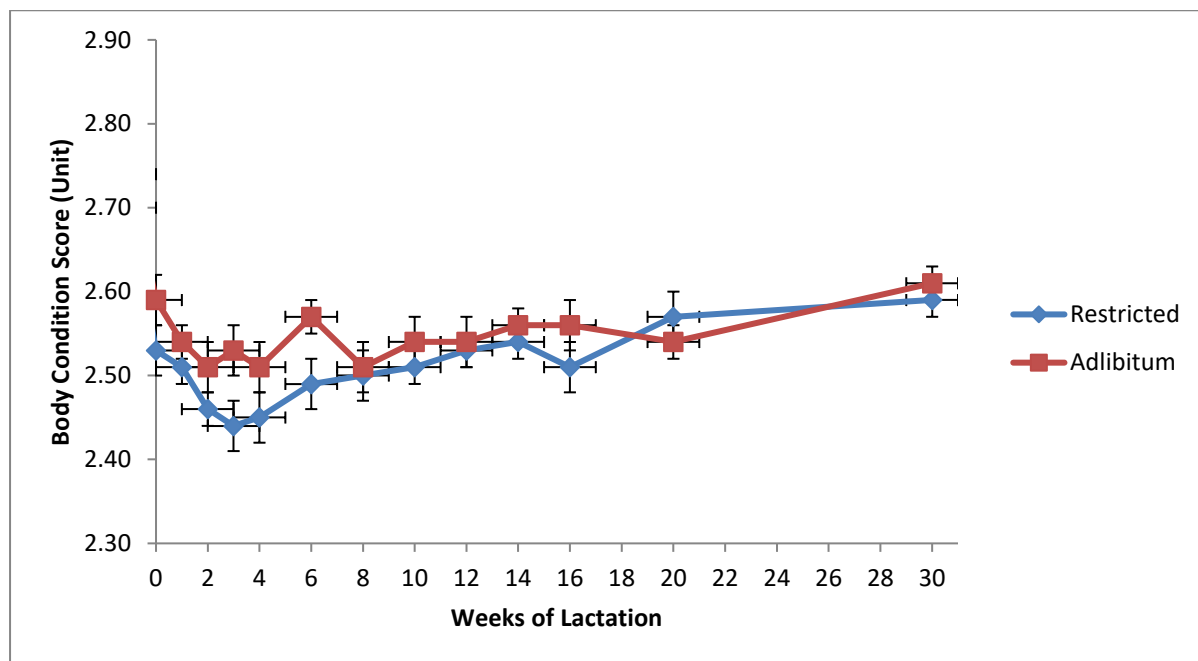


Figure 3.4 Changes in body condition score by weeks of lactation for Group A (*ad libitum*) and Group R (restricted). Values presented as mean \pm SEM. Body condition score on the day of calving was not different (Group A: 2.59 ± 0.03 units *versus* Group R: 2.53 ± 0.03 units), ($P=0.196$).

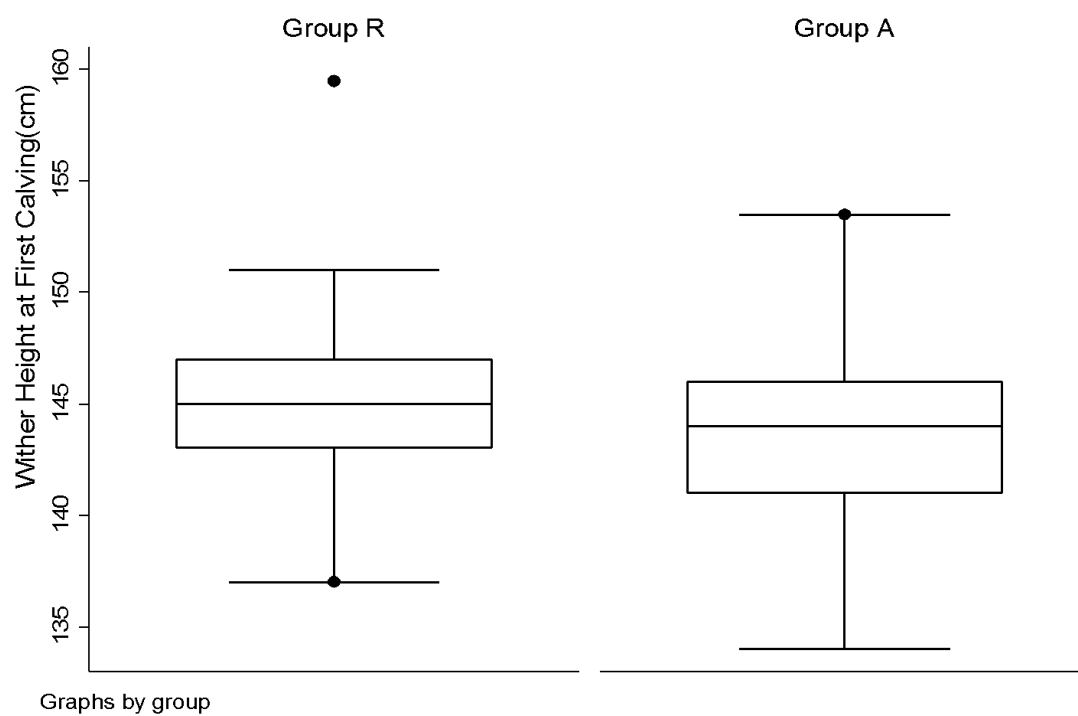


Figure 3.5 Box plot for wither height on the day of calving for Group A (*ad libitum*) (n=39) and Group R (restricted) (n=37), (P=0.076).

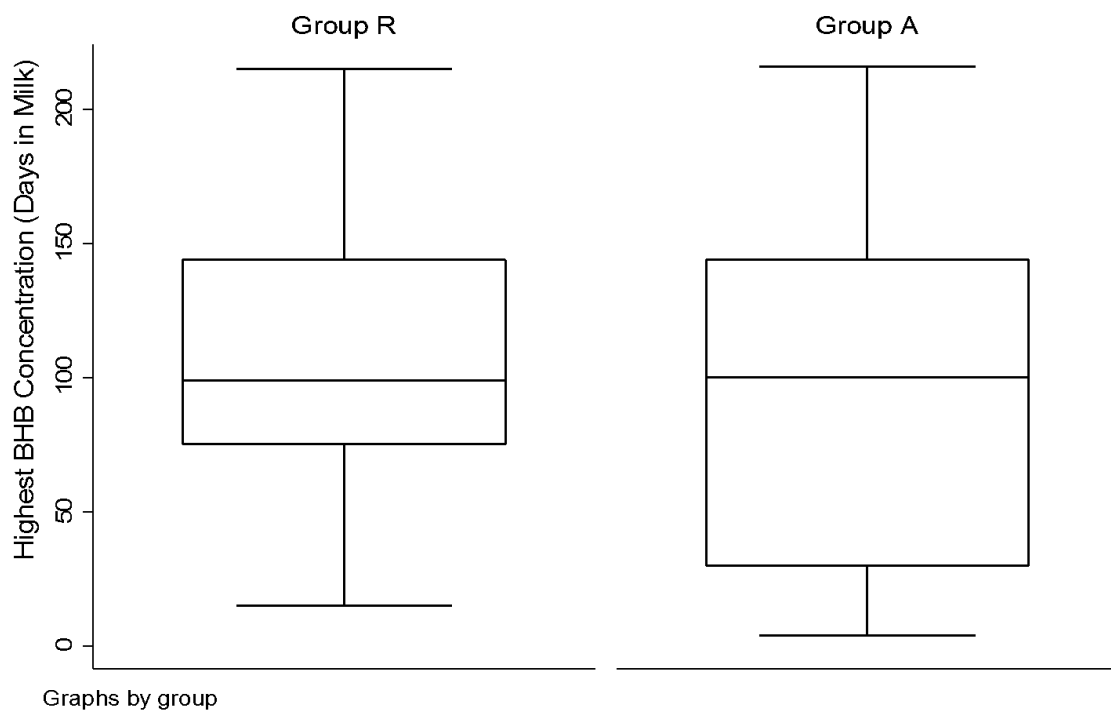


Figure 3.6 Box plot for DIM for peak BHB concentration between Group A (*ad libitum*) (n=43) and Group R (restricted) (n=39), (P=0.433).

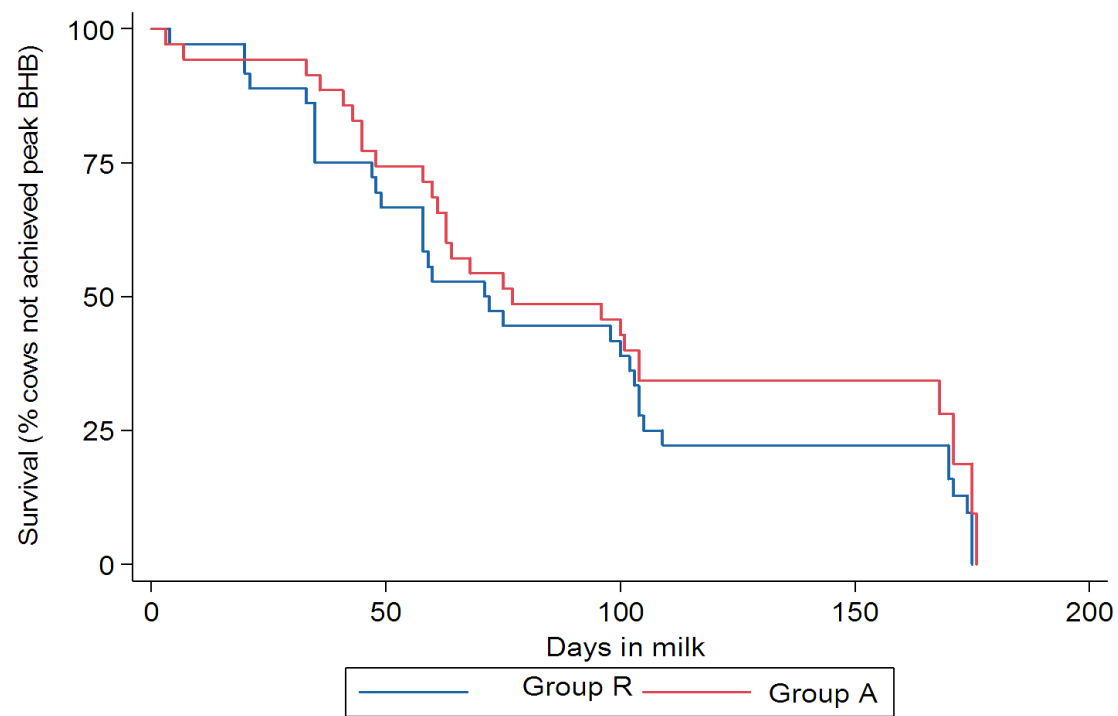


Figure 3.7 Kaplan-Meier survival curve for percentage of cows were not achieving peak BHB concentration by group. Median time to event was 77 days, n=43 for *ad libitum* (Group A) and 71 days, n=39 for restricted (Group R), respectively (P=0.209, log rank test).

3.3.1.1 Relationships among Variables between Dam (Heifer) and Day Old Calf

Heart girth, crown rump length, hock fetlock length and wither height of the dam showed a significant positive correlation with heart girth, crown rump length, hock fetlock length and wither height of her calf ($r=0.38$; $P=0.002$), ($r=0.50$; $P<0.050$), ($r=0.52$; $P<0.050$) and ($r=0.25$; $P=0.041$), respectively. However, belly girth and loin height of the dam were not significantly correlated with belly girth and loin height of the calf ($r=0.18$; $P=0.148$) and ($r=0.18$; $P=0.143$), respectively.

AFC showed a significant positive correlation with BW at calving ($r=0.45$; $P<0.050$).

BW also showed a significant positive correlation with HG ($r=0.79$; $P<0.050$).

3.3.1.2 Body Weight

Actual changes in body weight and BCS over time are shown in Figure 3.3 and Figure 3.4 respectively. The following variables: pre-weaning dietary group (*ad libitum* milk replacer versus restricted milk replacer), DIM, BCS, BCS change, BHB concentration values, wither height, rumen fill score and milk yield remained in the final regression model for postpartum body weight. Heifer identity accounted for 88.6% (95% CI 84.8 – 91.6%) of the residual variance as shown by the Intra-class correlation. Pre-weaning dietary group was retained in the final model since it was the explanatory variable of interest (Table 3.1).

There were significant changes in postpartum body weight with animals in both groups tending to lose weight over the first 3-4 weeks of lactation then gaining weight thereafter until the study end with animals gaining between approximately 50-70 kg during this period (Figure 3.3). Milk yield was positively associated with postpartum body weight, ($P=0.031$) as were BCS ($P<0.050$) and rumen fill score ($P<0.050$). There was a positive association

between β -hydroxybutyrate and postpartum body weight, ($P < 0.050$). However, there was no significant association between postpartum body weight with pre-weaning dietary group, ($P = 0.879$).

Table 3.1 Fractional polynomial regression model for association between postpartum body weight on a specific day postpartum and pre-weaning dietary group, DIM, BCS, BCS change, β -hydroxybutyrate concentration values, wither height, rumen fill score and milk yield measured on that day postpartum; with cow identity included as a random intercept and DIM as a random slope.

Outcome Variable: Postpartum Body Weight (kg)	Coefficient	[95% Confidence Interval]		P Value
Pre-weaning dietary group (A vs R)	1.23	-14.61	17.07	0.879
DIM	-25.68	-29.41	-21.95	≤ 0.001
DIM ^{0.5}	11.99	10.66	13.32	≤ 0.001
BCS (unit)	59.38	47.97	70.78	≤ 0.001
BCS change since previous sample (unit)	-8.96	-17.55	-0.37	0.041
Rumen fill score (unit)	10.26	7.35	13.18	≤ 0.001
β -hydroxybutyrate (mmol/L)	0.84	0.51	1.18	≤ 0.001
Milk yield (kg)	0.21	0.02	0.39	0.031
Wither height (cm)	0.54	-0.17	1.26	0.135
Constant	374.28	342.61	405.94	≤ 0.001

Random-effects Parameters	Estimate	[95% Confidence Interval]	
Heifer identity:			
DIM	0.15	0.12	0.18
Constant	36.64	31.27	42.93
Residual error	13.12	12.53	13.74

Residual Intraclass Correlation (ICC)

Level	ICC	[95% Confidence Interval]	
Heifer identity	0.886	0.848	0.916

3.3.1.3 *Beta-hydroxybutyrate*

The following variables: pre-weaning dietary group (*ad libitum* milk replacer versus restricted milk replacer), DIM, body weight, BCS, BCS change and milk yield were remained in the final regression model for postpartum BHB concentration (Table 3.2). Heifer identity accounted for 23.5% (95% CI 16.0 – 33.3%) of postpartum BHB concentration residual error. Pre-weaning dietary group was retained in the final model since it was the primary explanatory variable of interest.

There was significant variation in postpartum BHB concentration with DIM on which it was measured, ($P < 0.050$); body weight (0.02 kg 95% CI 0.01 – 0.03 per kg increase), ($P < 0.050$). The impact of BCS ($P = 0.053$) and BCS change ($P = 0.072$) on postpartum BHB was complex with best model fit achieved by inclusion of a BCS* BCS change interaction term, ($P = 0.048$). However, there were no significant association between postpartum BHB concentration with pre-weaning dietary group, ($P = 0.179$) and milk yield, ($P = 0.340$).

Table 3.2 Fractional polynomial regression model for association between postpartum BHB concentration on a specific day postpartum and pre-weaning dietary group, DIM, body weight, BCS, BCS change, interaction BCS-BCS change and milk yield; with cow identity was included as a random intercept with DIM as a random slope.

Outcome Variable: Postpartum BHB Concentration (mmol/L)	Coefficient	[95% Confidence Interval]		P Value
Pre-weaning dietary group (A vs R)	-0.47	-1.15	0.21	0.179
Log [DIM]	1.95	1.41	2.48	≤0.001
DIM ^{0.5}	0.00	0.00	0.00	≤0.001
DIM	-0.15	-0.20	-0.10	≤0.001
Body weight (kg)	0.02	0.01	0.03	≤0.001
BCS (unit)	-1.69	-3.40	0.02	0.053
BCS increase (unit) since previous sample	-15.28	-31.95	1.38	0.072
Interaction BCS*BCS change	6.68	0.07	13.29	0.048
Milk yield (kg)	-0.01	-0.05	0.02	0.340
Constant	-4.29	-8.67	0.09	0.055

Random-effects Parameters	Estimate	[95% Confidence Interval]	
Heifer identity			
DIM	0.00	0.00	0.00
Constant	1.89	1.18	3.03
Residual error	6.14	5.61	6.72

Residual Intraclass Correlation (ICC)

Level	ICC	[95% Confidence Interval]	
Heifer identity	0.235	0.160	0.333

3.3.1.4 Subclinical Ketosis (SCK)

The following variables: pre-weaning dietary group (*ad libitum* milk replacer versus restricted milk replacer), interaction milk yield-DIM, BCS, BCS change and body weight were remained in the final logistic regression model for occurrence of SCK defined as a BHB concentration > 1.2 mmol/l (Table 3.3). Heifer identity accounted for 25.0% (95% CI 13.9 – 40.7%) of SCK residual error. Pre-weaning dietary group was retained in the final model since it was the primary explanatory variable of interest.

There were significant association between SCK and milk yield (1.08 95% CI 1.02 – 1.13), (P=0.008); DIM (1.02 95% CI 1.00 – 1.03), (P=0.018) and body weight (1.01 95% CI 1.00 – 1.02), (P=0.002). However, there were no significant association between SCK and pre-weaning dietary group, (P=0.245); BCS, (P=0.066); BCS change, (P=0.075) and interaction milk yield-DIM, (P=0.071).

Group R heifers were more likely to have a single measurement of BHB over 1.2 mmol/l suggesting SCK compared to Group A heifers, (P=0.040) Table 3.4.

Table 3.3 Multivariable logistic regression model for association between occurrence of SCK and pre-weaning dietary group, interaction milk yield-DIM, BCS, BCS change and postpartum body weight on a specific day postpartum; with cow identity was included as a random effect.

Outcome Variable: Subclinical Ketosis (BHB > 1.2 mmol/L)	Odd Ratio	[95% Confidence Interval]		P Value
Pre-weaning dietary group (A vs R)	0.68	0.36	1.30	0.245
Milk yield (kg)	1.08	1.02	1.13	0.008
DIM (days)	1.02	1.00	1.03	0.018
Interaction milk yield-DIM	0.99	0.99	1.00	0.071
BCS (unit)	0.14	0.02	1.14	0.066
BCS change (unit) since previous sample	5.55	0.84	36.49	0.075
Body weight (kg)	1.01	1.00	1.02	0.002
Constant	0.00	0.00	0.08	0.003

Random-effects Parameters	Estimate	[95% Confidence Interval]	
Heifer identity			
Constant	1.05	0.73	1.50

Residual Intraclass Correlation (ICC)

Level	ICC	[95% Confidence Interval]	
Heifer identity	0.250	0.139	0.407

Table 3.4 Incidence of SCK for Group A (*ad libitum*) and Group R (restricted).

Group A n (%)	Group R n (%)	P value
26/46 (56.5%)	31/40 (77.5%)	0.040

3.3.1.5 Time to Peak BHB Concentration

There was no difference in DIM when peak BHB concentrations were achieved between Group A and Group R heifers. The median DIM for peak BHB concentration for Group A was (100.0 days) [interquartile range (IQR): 30.0 to 144.0 days] and Group R was (99.0 days) (IQR: 75.0 to 144.0 days), ($P=0.433$) (Figure 3.6). Whilst there was no significant difference in median survival time, ($P=0.209$) appraisal of Figure 3.7.

Cox proportional hazard modelling was employed to investigate potential explanatory variables associated with time to peak BHB concentration. Univariable Cox models were fitted for the following explanatory variables: Pre-weaning feeding group (A *versus* R), body weight, body weight change, BCS, BCS change, rumen fill score and age; (Table 3.5).

However, there were no significant associations between time to peak BHB concentration with pre-weaning dietary group, body weight, BW change, BCS, BCS change, rumen fill score and age of the heifers.

Table 3.5 Univariable Cox regression model with time to peak BHB, explanatory variables, hazard ratio, 95% confidence interval and p-value.

Explanatory variable	Hazard Ratio	[95% Confidence Interval]		P-Value
Group (A vs R)	0.75	0.46	1.24	0.266
Body weight (kg)	1.00	0.99	1.01	0.784
BW change since previous sample	1.00	0.98	1.01	0.769
BCS (unit)	2.46	0.21	28.22	0.470
BCS change since previous sample	1.53	0.11	20.35	0.748
Rumen fill score (unit)	0.47	0.21	1.07	0.071
Age (days)	1.00	0.99	1.01	0.631

3.3.2 Calf Measurement

Descriptive Statistics

Birth Weight of Calf

There was no significant difference in calf birth weight between Group A and Group R heifers (Group A calf: 41.3 ± 0.9 kg *versus* Group R calf: 42.1 ± 1.0 kg), ($P=0.585$) (Figure 3.8). However, regardless of group, bull calves weighed significantly more than heifer calves 44.0 ± 1.2 kg *versus*: 39.9 ± 0.7 kg, respectively ($P=0.002$) (Figure 3.9).

Heart Girth (HG) of Calf

There was no significant difference between the 2 groups with respect to HG of calves born (Group A calf: 79.0 ± 0.6 cm *versus* Group R calf: 78.8 ± 0.7 cm), ($P=0.854$).

Belly Girth (BG) of Calf

There was no significant difference between groups with respect to BG of calves born (Group A calf: 81.4 ± 0.7 cm *versus* Group R calf: 81.0 ± 0.8 cm), ($P=0.722$).

Wither Height (WH) of Calf

There was no significant difference in WH of calves born to Group A and Group R heifers (Group A calf: 76.6 ± 0.5 cm *versus* Group R calf: 76.4 ± 0.6 cm), ($P=0.770$) (Figure 3.10).

Loin Height (LHt) of Calf

There was no significant difference between LHt of calf born to Group A and Group R heifers (Group A calf: 81.1 ± 0.6 cm *versus* Group R calf: 80.6 ± 0.5 cm), (P=0.538).

Crown Rump Length (CRL) of Calf

There was no significant difference between groups with respect to CRL of calves (Group A calf: 91.0 ± 1.2 cm *versus* Group R calf: 92.7 ± 1.1 cm), (P=0.330).

Hock Fetlock Length (HFL) of Calf

There was no significant difference in HFL between calves from Group A and Group R heifers (Group A calf: 39.0 ± 0.3 cm *versus* Group R calf: 39.4 ± 0.4 cm), (P=0.354).

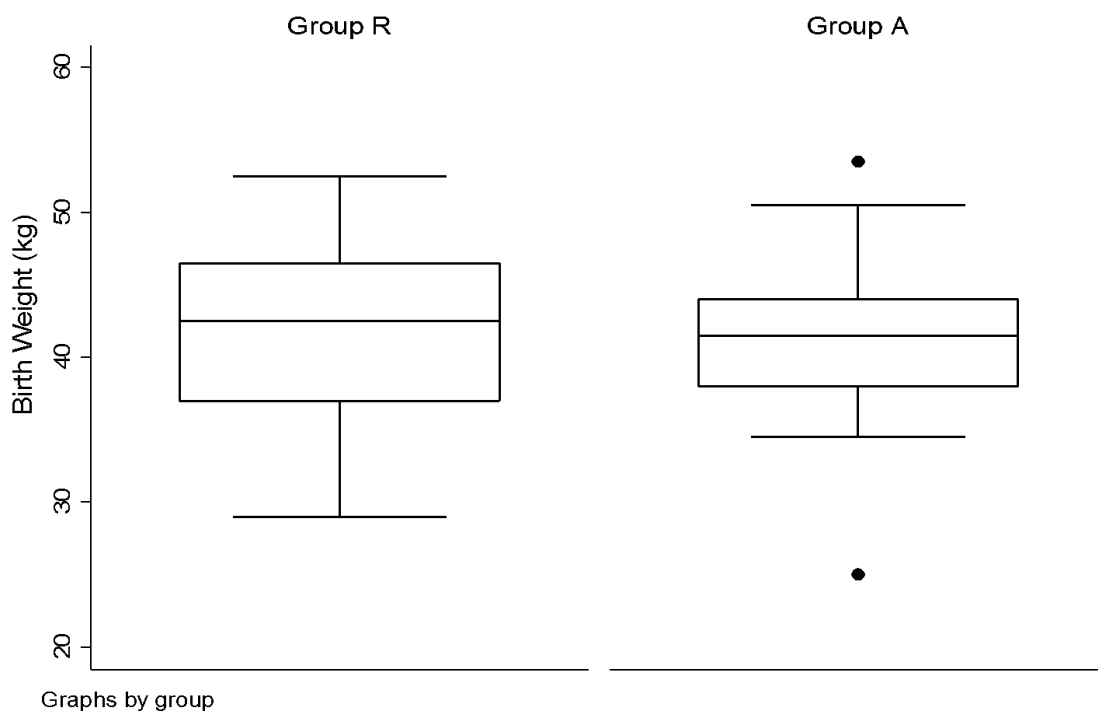


Figure 3.8 Box plot for calf birth weight for Group A (*ad libitum*) (n=35) and Group R (restricted) (n=35), (P=0.585).

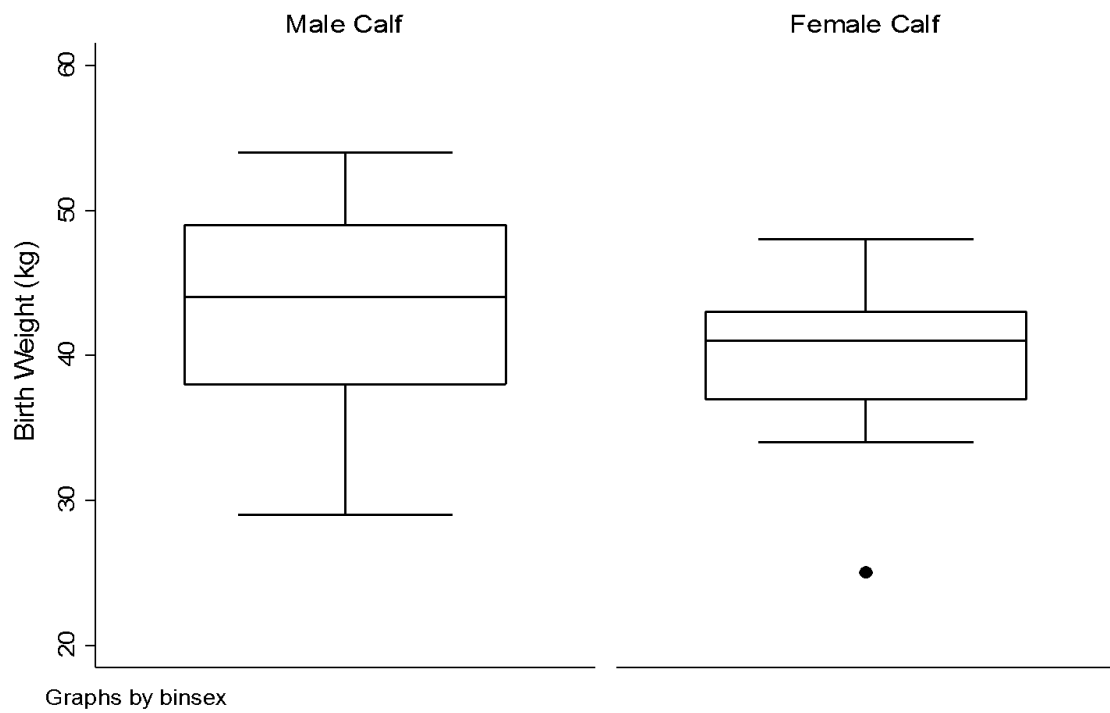


Figure 3.9 Box plot for birth weight for bull calf (n=32) and heifer calf (n=39), (P=0.002).

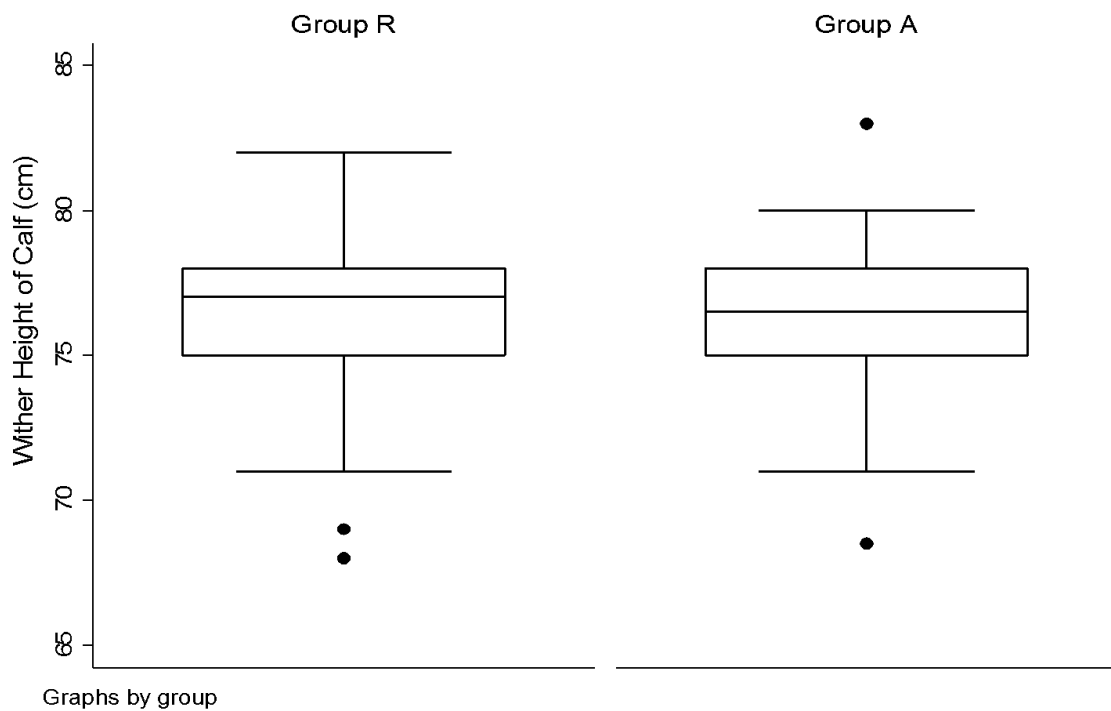


Figure 3.10 Box plot for wither height of calf for Group A (*ad libitum*) (n=34) and Group R (restricted) (n=34), (P=0.770).

3.4 Discussion

In the present study, AFC, BW and BCS of heifers on the day of calving were not different between Group A and Group R. To the best of our knowledge, the present study is the first to evaluate and compare above parameters in heifers that received different milk replacer during pre-weaning period.

3.4.1 Age at First Calving (AFC)

Despite a tendency for a lower age at first observed oestrus and an earlier conception in Group A, our findings, agree with the findings of a number of other studies that have compared traditional and accelerated pre-weaning nutritional regimes (Aikman et al., 2007; Drackley et al., 2007; Morrison et al., 2009; Terre et al., 2009). In contrast, other studies do find a 28 to 31 days reduction in AFC with heifer calves that were offered a higher feed level up to weaning (Bar-Peled et al., 1997; Raeth-Knight et al., 2009).

AFC influences milk yield and milk composition (Pirlo et al., 2000). Reduction of AFC has a positive effect on genetic progress because the generation interval decreases and progeny tests of sampling bulls are carried out earlier (Pirlo et al., 2000). Gardner et al. (1988) stated that reducing in AFC can reduce feed costs and allow an earlier return on investment.

Average AFC has not been reduced during the last decades because of the belief that early calving is harmful to milk yield and longevity (Pirlo et al., 2000). Reduction of AFC can increase the number of calves per cow, but dystocia can be an inhibiting factor that may reduce the viability of calves (Martinez et al., 1983). Dystocia may cause the calves susceptible to infections because of reduced absorption of immunoglobulins (Donovan et al., 1986). Simerl et al. (1991) reported that frequency of dystocia to be greater in both older (>27

months) and younger heifers (<22 months). Although reduction of AFC is one of the most effective strategies for reducing replacement costs, many dairy farmers remain sceptical of calving heifers at ages less than 24 months (Pirlo et al., 2000).

3.4.2 Body Weight (BW)

In our study, body weights of heifers on the day of calving (569 kg) were heavier than previously reported for example, 524 kg by Taylor et al. (2004). Two studies conducted by Heinrichs (1993) and Tozer and Heinrichs, (2001) recommended that for modern Holstein heifers average AFC is ≤ 24 months with a body weight >560 kg after calving to maximise lactation performance and to reduce rearing costs. Our results regarding body weight support this recommendation.

In the present study, a decreased in mean BW was observed from a week of calving until three weeks after calving and BW increased slowly during the subsequent lactation period in both groups (Figure 3.3). Body weight alone is not a good indicator of body reserves, as the relationship is affected by factors such as breed, frame size, gestation, parity and stage of lactation (Enevoldsen and Kristensen, 1997; Berry et al., 2006b). BW changes do not completely reflect changes in adipose and lean tissue weight (NRC, 2001). In fact, tissue mobilisation in early lactation occurs in spite of animals increasing their dry matter intakes, since this increased DMI alone is insufficient initially to provide sufficient energy to keep up with the demands of lactation (Berry et al., 2006b; Roche et al., 2007). However, decreases in body tissue weight can be masked by increasing gastrointestinal fill thus mitigating apparent weight loss.

In our study; milk yield, BCS, BCS change, BHB concentration, DIM and rumen fill score were significantly associated with postpartum body weight. Positive relationships between first lactation milk yield and body weight at first calving have been previously reported in several studies (Clark and Touchberry, 1962; Fisher et al., 1983; Keown and Everett, 1986). Keown and Everette (1986) showed that first lactation milk yield increased as body weight increased up to 635 kg. A survey by Heinrichs and Hargrove (1987) on Holstein herds showed that milk yield was higher in herds for which the calving age was less than 26 months and postpartum body weight was greater than 520 kg.

Although, there is a moderate correlation between BCS and body weight ($r=0.55$; Berry et al., 2006a), associations between body weight and milk yield are unlikely to be the same as between BCS and milk yield, because body weight is also associated with maintenance requirements (a greater body weight requiring more energy for maintenance) and body weight change is often attenuated due to changes in gastrointestinal fill postpartum (NRC, 2001).

In our study, there were no significant association between postpartum body weight of the heifers with wither height (WH) and pre-weaning dietary group. The average WH on the day of calving (145 cm) was not different between Group A and Group R heifers. A suggested standard WH is 138 to 141 cm (Heinrichs and Hargrove, 1987; Hoffman, 1997). BW and WH gains of the milk replacer heifers were similar during the rearing period to the targeted points except for the postpartum BW (Kertz et al., 1998). WH gain at 12 months of age, which is expected to be 75% of adult height (Kertz et al., 1998). The postpartum BW to WH ratio was smaller for the milk replacer heifers in the study of Kertz et al. (1998), where they were heavier but not taller.

3.4.3 *Body Condition Score (BCS)*

In the present study, BCS of heifers on the day of calving (2.59) was lower compared to report for thirty two Holstein Friesian UK herds of 2.80 by Taylor et al. (2004). Cows that are over conditioned at calving ($BCS \geq 3.50$) will be compromised as they have reduced dry matter intake (DMI) just prior to calving and take longer to increase DMI during postpartum. They tend to have greater fat mobilisation and therefore, severe NEB in early postpartum than cows with an optimum BCS at calving (Roche et al., 2009). Therefore, it is recommended that cows have a BCS of 2.75 - 3.00 at calving and they are managed to have a BCS loss not more than 0.5 between calving and first service (Crowe, 2008).

In the present study, a decreased in mean BCS was observed from a week of calving until three weeks after calving and BCS recovered slowly during the subsequent lactation period in both groups (Figure 3.4). The general pattern of body condition change during early weeks of lactation period in this study was consistent with previous studies (Waltner et al., 1993; Ruegg and Milton, 1995). This is because high milk producing dairy cows experiences a substantial increase in energy requirements to facilitate the dramatic increases in daily milk yield, which peaks between 4 and 8 weeks of postpartum. This requirement is only partially met by increased feed consumption (due to limitations in intake and appetite) and the remainder being met by mobilisation of body reserves resulting in cows entering negative energy balance (NEB) (Grummer, 2007). The consequences of severe NEB are an increased risk of metabolic diseases, which occur within the first month of lactation, reduced immune function and a reduction in fertility (Roche et al., 2009). Mobilisation of body fat during NEB increased plasma concentrations of BHB and NEFA, which were associated with reduced fertility (Garnsworthy et al., 2008). However, in our study there was very low incidence of metabolic diseases and reproductive disorders (Chapter 4).

3.4.4 Time to Peak BHB Concentration

From our study, we found that there were no association between time to peak BHB concentration with pre-weaning dietary group, BW, BW change, BCS, BCS change, rumen fill score and age of the heifers.

3.4.5 Subclinical Ketosis (SCK)

To the best of our knowledge, the present study is the first to show association of the likelihood of SCK in heifers and pre-weaning nutrition. In our study, restricted milk replacer fed heifers were more likely to have a single measurement of BHB over 1.2 mmol/L suggesting SCK compared to *ad libitum* milk replacer fed heifers. There was also a significant association between SCK with body weight, milk yield and DIM. Our data also showed that peak prevalence of SCK occurred at 14 weeks of postpartum between the 2 groups. In contrast to our results, other studies (McArt et al., 2012; Vanholder et al., 2015) reported that the peak prevalence of SCK occurred within the first week of postpartum. More than 90% of SCK cases occur during the first and second months of postpartum, with the former containing the peak prevalence (Duffield et al., 1997; Suthar et al., 2013). In the first 65 DIM, the prevalence of SCK may range from 7 to 34% with considerable between herd and study variation (Duffield et al., 2009; McArt et al., 2012; Suthar et al., 2013).

In the present study, Group R heifers were more susceptible to SCK incidence compared to Group A heifers (Table 3.4). It can be speculated that influence of epigenetic may be involved. The term of epigenetic was proposed by Waddington in 1940, to describe the interaction of genes with the environment. The environment can influence gene expression in many different ways. Some environmental conditions generate immediate and short lived

changes in gene expression while others generate long-lasting effects. Even though the influence of epigenetic mechanisms was not studied, the outcome of such event has been observed (Robison et al., 1988). In the literature, the time of highest prevalence of SCK would depend on several factors related to the nutritional management and genetic aspects (Uribe et al., 1995). Furthermore, different studies showed that the prevalence of SCK during the first 2 months of lactation was highly variable between herds (Dohoo & Martin, 1984; Oetzel, 2007) and has been associated with differences in genetics and management (Ostergaard & Tind Sorensen, 1998). In the other studies, conclude that approximately 50% of all lactating cows will go through a stage of subclinical ketosis in early lactation (Erb and Grohn, 1988; Duffield, 2000).

We also found that there were no association between SCK and BCS in our study. It may be speculated that majority of our heifers' BCS range between 2.50 – 2.75 during postpartum period. However, over-conditioned cows have been reported to be more at risk of both SCK and clinical ketosis (CK) in the transition period (Duffield et al., 2001; Gillund et al., 2001; McArt et al., 2013).

Peak incidence occurred in the first week of postpartum. Important risk factors for subclinical ketosis have been reviewed (Duffield, 2000). The predominant factors were identified include body condition, parity, herd, genetic and season. BCS prior to calving is an important risk factor for subsequent development of subclinical ketosis during lactation (Gearhart and Curtis, 1990; Heuer et al., 1999). Cows at BCS ≥ 4.0 were at the highest risk and had the highest BHB concentrations compared to normal and thin cows prior to calving. Genetic predisposition, breed and seasonal differences account for some of the variation in subclinical ketosis rates.

3.4.6 *Birth Weight of Calves*

Maternal nutrition during gestation has been associated with changes in calf birth weight in some but not all studies (Holland and Odde, 1992; Greenwood and Cofe, 2007). In the current study, calf birth weight was not affected by the different pre-weaning feeding strategy during early life of their dam and to the best of our knowledge; this study is the first to evaluate the effect of different pre-weaning nutrition in heifers. Rasby et al. (1990) suggested that major reductions in dam nutrient intake are needed to affect calf birth weight. Dams could have adapted their metabolism to the nutritional input to supply enough nutrients for the developing foetus. Calf birth weight was not different between Group A and Group R heifers. However, bull calves were heavier compared to heifer calves. This has been observed in other studies (Kertz et al., 1997; Dhakal et al., 2013). Furthermore, male calves are carried 1.3 day longer than female calves, which may explain their higher birth weight (Dhakal et al., 2013). Thus, sex specific genes affecting insulin sensitivity, for example mutations in the glucokinase gene, may be responsible for the sex difference in birth weight. Genetically more insulin resistant female foetus is less responsive to the trophic effects of insulin and therefore, lighter (Wilkin and Murphy, 2006). Maternal nutrition may influence the birth weight of calves. A study carried out in manipulation of diet during early to mid-gestation influenced the birth weight of calves from beef heifers dams (Micke et al., 2010). However, a different study has showed that the birth weight of a calf was not influenced by the diet of the dam unless there was significant long term nutritional deficiency (Holland and Odde, 1992).

Size at birth is an indicator of foetal growth which is primarily determined by the maternal uterine environment. Nutrients supplementation to the developing foetus is influenced by body condition at conception (McCrabb et al., 1992), maternal age, maternal food supply during pregnancy (Osgerby et al., 2004) and also the size of the placenta (McMillen et al.,

2001). Thus the lack of a difference in calf birth weight between treatments suggest treatment had not effect of uterine blood flow or environment.

3.5 Conclusion

Different milk replacer feeding strategies applied during early life of heifer calves in the present study did not have effect on physical growth of the heifers and also their offspring performance. Meanwhile, AFC was not affected but Group R heifers were more prone to SCK incidence that occurred at 14 weeks of postpartum.

Calf birth weight did not differ between the groups. It is clear that under the conditions of this study, different pre-weaning feeding strategy of the heifer calves did not compromise their calf physical performance potential.

Chapter 4

Effect of Different Pre-Weaning Feeding Strategies in Early Life of Heifer Calves on Fertility and Health Performances

4.1 Introduction

Fertility of the dairy cows has been declining over the last 30 years while milk yield has been increasing (Lucy, 2001). Royal et al. (2000) reported that there was a decline in pregnancy rate to first artificial insemination (AI) after calving of 1% per year during the period from 1975 to 1998 in the United Kingdom. Currently, the issue of reduced fertility in dairy herds is one of the most important factors that affecting dairy producer profitability. Roche (2006) has identified that the effect of infertility on dairy farmers profitability as (i) prolonged calving interval with fewer calves and less milk per cow per year; (ii) increased replacement costs; (iii) increased labour, semen and veterinary bills and (iv) an extended low production or dry period which increases body condition score (BCS) at calving and reduces fertility at the subsequent breeding season.

Neonatal nutrition influences performance in later life in many species (Desai and Hales, 1997), however, there has been little study of this phenomenon in cattle. Determining if there are direct and residual effects of nutrition during the first 60 days of life may help optimise lifetime productivity (Drackley and Bartlett, 2001). Underfeeding reduces calf weaning weight and delays puberty (Short and Bellows, 1971), which reduces the potential lifetime productivity of the dairy heifers. Several studies found a positive relationship between level of nutrition in early life and subsequent milk production (Shamay et al., 2005; Moallem et al., 2006; Drackley et al., 2007). A 31-day reduction in first calving age (Bar-Peled et al., 1997) and a 23-day reduction in age at puberty (Shamay et al., 2005) have been observed when heifer calves were given a higher feed level up to weaning. Conversely, Aikman et al. (2007) observed no effects of increasing the level of nutrition during the pre-weaning period on first lactation milk production.

The ability of a dairy cow to reproduce is fundamental for milk production, the dairy cow, the dairy farmer and thus, for the whole dairy industry. The optimal calving interval is generally considered to be about 12 months from an economic point of view, meaning that the cow should be bred and conceived 3 months after calving (Petersson et al., 2008). Otherwise, more services per conception may be required, calve later, leading to decreased milk production and later an increased risk of the cow being culled (Wathes et al., 2008). However, De Vries (2006) calculated the cost benefit of a pregnancy at different times post calving and stated that if breeding was delayed when the value of pregnancy was negative and that optimal breeding decisions for individual cows were greatly dependent on the predicted daily milk yield for the remaining period of lactation.

Many studies have used milk progesterone analysis to assess and monitor reproductive function during the postpartum period. Evaluation of milk progesterone profiles can offer an objective method for the characterisation of the postpartum ovarian activity in dairy and beef cows. A normal profile consists of a period of low progesterone after luteolysis of the corpus luteum of pregnancy at the time of birth followed by increasing concentrations that are indicative of the first postpartum ovulation. Then, a period of falling and rising progesterone, reflecting ovarian cyclical activity, follows until the maintenance of luteal function to support pregnancy (Lamming and Bulman, 1976). Deviations from a normal progesterone profile were associated with decreased fertility in the dairy cow (Bulman and Wood, 1980). Early commencement of luteal activity (CLA) after calving has been associated with increased probability for early service, shorter interval between calving and conception, higher conception rate and lower number of services per conception (Darwash et al., 1997a). It has also been reported that delayed first ovulation and cessation of cyclical activity increase the calving interval (Petersson et al., 2006a) and decrease the pregnancy rate to first AI (Royal et al., 2000).

At present, there are limited studies and data concerning the impact of early life nutrition of ruminants on reproductive performance of their female offspring. Corah et al., (1975) reported that age at puberty of heifer calves from energy restricted primiparous dams was increased by 19 days, but pregnancy rate to each service of the heifer calves was not measured in this study. The supply of nutrients to the developing foetus is influenced by maternal age and body condition score at conception (McCrabb et al., 1992) and maternal food supply during pregnancy (Osgerby et al., 2004).

Postpartum nutrition plays a significant role in the onset of ovarian cyclicity, the expression of normal oestrous cycles and conception rates (Robinson et al., 2006). In general, an increase in milk energy output cannot initially be matched by a proportionate increase in energy intake, resulting in a negative energy balance (NEB), which forces the mobilisation of body reserves (Gilmore et al., 2011). Negative energy balance has been identified as an underlying causal factor of poor reproductive performance in high-yielding dairy cows (Jorritsma et al., 2003) and has been associated with a delay in the CLA (Jolly et al., 1995), an extended interval to first service (Butler et al., 1981) and reduced conception rates (Domecq et al., 1997). NEB also affects ovarian function through a decrease in the maximum diameter of dominant ovarian follicles (Lucy et al., 1991; Mackey et al., 1999). Smaller dominant follicles produce less oestradiol, suppressing the pulsatile secretion of luteinising hormone (LH) (Butler, 2001) and decreasing ovarian responsiveness to LH (Butler, 2001). Therefore, these situations increase the proportion of follicles that fail to ovulate (Mackey et al., 1999).

The aims of the present study were to determine the effect of milk replacer feeding strategy in early life of heifer calves on their future fertility and health performance by comparing the fertility and health parameters between *ad libitum* milk replacer fed calves (Group A) and restricted milk replacer fed calves (Group R).

4.2 Materials and Methods

4.2.1 Animals

The present study was conducted from 4th April 2013 until 16th April 2015 on 88 pedigree Holstein heifers at Wood Park Farm, University of Liverpool, United Kingdom. Heifers in Group A – *ad libitum* (forty seven animals) and Group R – restricted (forty one animals). They were between 23-32 months of age. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 under a UK Home Office licence for work on living animals and with the approval of the University of Liverpool Ethical Review Process.

4.2.2 Vaginal Mucus Score

During postnatal checks (PNC) at 35-42 days post calving, samples of vaginal mucus were collected in a gloved hand and assessed for colour, proportion and volume of pus and a character score was assigned as follows: (0) clear or translucent mucus; (1) mucus containing flecks of white or off-white pus; (2) < 50 ml exudate containing $\leq 50\%$ white or off-white mucopurulent material; and (3) > 50 ml exudate containing $\geq 50\%$ purulent material, usually white or yellow, but occasionally sanguineous following the method of Williams et al. (2005) by one of the three experienced veterinary surgeons employed by University of Liverpool Farm Animal Practice.

4.2.3 Fertility Parameters

Voluntary waiting period for this farm was 50 days post calving. Oestrus was detected using a combination of behavioural observations and electronic aids namely: IceQube data loggers

(IceRobotics Ltd, Edinburgh, UK) attached to the hind leg and programmed to record cows' lying time activity. Collar tags (SCR Heetime) attached to the left side of each cow's neck with a strap. The collar contains a microphone that was able to record the distinctive sounds of regurgitation and rumination. Data were calculated and summarised in 2 hours interval and stored in the memory of the logger (Data Flow software, SCR Engineers Ltd). All required data were transferred to receiver units that were installed in the drinker areas of straw yard and milking parlour and then sent to the management software on the farm computer. Cows were artificially inseminated at detected oestrus. Pregnancy diagnosis was performed approximately 30 days after artificial insemination (AI) by per rectum ultrasonographic examination of the uterus by experienced veterinary surgeons. Data for the period from calving to first oestrous, calving to first service, days to conception from the start of the designated service period or from calving date, number of serves required and calving interval were recorded in InterHerd software (PAN Livestock Services Ltd).

4.2.4 *Hormone Assays*

Progesterone was analysed as pregnane metabolites in 50 μ L whole milk samples using an established enzyme immunoassays (EIA) (Walker et al., 2008). For assays included in this dataset, the intra- and inter-assay coefficients of variation were 9.7% (at 1.85 ng/ml \pm 0.18 ng) and 13.0% (at 0.22 ng/ml \pm 0.03 ng) respectively.

4.2.5 *Health*

Calving ease was scored on a categorical scale designed to be practical as follows: (1) normal no assistance; (2) minor manual assistance and (3) required considerable assistance (Heinrichs and Heinrichs, 2011).

Presence of a retained foetal membranes (RFM) was defined as the failure to expel the placenta within 12 hours of calving (Eiler, 1997). It was assessed by experienced farm staff and recorded on the InterHerd computerised farm recording system.

Animals were observed and assessed for mobility using the 0-3 DairyCo mobility scoring system every 3 months (DairyCo, 2013). Incidence of lameness (score 2 and 3) requiring foot trimming or a findings of lameness at routine foot trimming at 60 days after calving and at drying off were recorded by the farm staff on InterHerd.

Cases of clinical mastitis were defined as presence of clots or watery milk, with or without inflamed teats. A cell count $<100,000$ cells/ml was classified as low somatic cell counts (SCC) and a count $\geq 100,000$ cells/ml was classified as high (Dohoo & Morris, 1993). Individual cow milk samples were collected monthly and analysed at the Cattle Information Service (CIS), Rickmansworth, UK, for fat percentage, protein percentage and somatic cell counts (SCC). Data were recorded on InterHerd by the farm staff.

Animals were observed and assessed for disease incidence during routine farm tasks and it was recorded by the farm staff on InterHerd. The University of Liverpool Farm Animal Practice were called to any ill cows to make a diagnosis.

4.2.6 *Classification of Pregnane Profiles*

Definitions for onset of ovarian activity postpartum and abnormal progesterone profiles have been given in several studies Darwash et al. (1997a), Royal et al. (2000) and McCoy et al. (2006). They are briefly summarised below. The onset of luteal activity postpartum was defined as the occurrence of two or more consecutive milk pregnane concentrations ≥ 0.3 ng/ml which suggests luteal phase concentrations of this metabolite (Holman et al., 2011). Postpartum interval to commencement of luteal activity (**CLA**) was defined as the first day postpartum on which milk pregnane concentrations were elevated ≥ 0.3 ng/ml. When measuring this interval, twice weekly sampling introduces an over-estimate of 1.75 days, on average and therefore the data were corrected by subtracting 1.75 days from the value obtained before analysis (McCoy et al., 2006). The luteal phase (**LP**) length of an individual oestrous cycle was defined as the time between the first elevated pregnane concentration measuring ≥ 0.3 ng/ml and the final consecutive milk pregnane concentration measuring ≥ 0.3 ng/ml. The inter luteal interval (**ILI**) was defined as the time between the demise of one corpus luteum and the rise of the next. It is the interval from the first milk pregnane concentration < 0.3 ng/ml at the end of one cycle (following luteolysis) to the last consecutive milk pregnane concentration < 0.3 ng/ml thus just before the next luteal phase.

Normal pregnane profile was defined as milk pregnane concentration > 0.3 ng/ml within 45 days postpartum and regular cycles thereafter with luteal phases of up to 19 days and inter luteal phases of less than 12 days (Taylor et al., 2003).

Abnormal pregnane profiles were defined as follows. Delayed ovulation type I (**DOV1**) was defined as milk pregnane concentration < 0.3 ng/ml for ≥ 45 days postpartum. Delayed ovulation type II (**DOV2**) was defined as milk pregnane concentration < 0.3 ng/ml for ≥ 12 days postpartum after CLA. Persistent corpus luteum type I (**PCL1**) was defined as milk

pregnane concentration ≥ 0.3 ng/ml for ≥ 19 days on the first luteal phase or delayed luteolysis of the corpus luteum during the first postpartum oestrous cycle. Persistent corpus luteum type II (**PCL2**) was defined as milk pregnane concentration ≥ 0.3 ng/ml for ≥ 19 days in a subsequent luteal phase.

4.2.7 Statistical Analysis

All data were analysed using Stata 13 (StataCorp) with data expressed as mean \pm SEM or proportions where appropriate. Data were compared between Group A and Group R. All data were checked for normality using normal quantile-quantile plots and basic descriptive statistics were carried out. Normally distributed data is described using mean values and standard errors of the mean. However, where data are not normally distributed, medians and inter quartile range (IQR) are presented.

Data for endocrine and traditional fertility parameters were analysed between groups using two-sample t-test and Kruskal-Wallis rank test or Wilcoxon rank-sum test (non-parametric tests). Frequency of different classifications of pregnane profiles (normal, DOV1, DOV2, PCL1 and PCL2) between groups were compared with chi-squared test.

Survival analysis was performed to evaluate time to commencement of luteal activity (CLA). Kaplan-Meier survival curves were plotted for Group A and Group R animals separately. Survival curves were compared between the 2 groups using the log-rank test.

Univariable Cox regression models were fitted to investigate the impact of pre-weaning feeding group (A *versus* R) and potential confounders on the time of CLA. Potential confounders considered were: average daily milk yield for weeks 1 to 8, change in body weight from calving to time of CLA, change in BCS from calving to time of CLA, and both

β -hydroxybutyrate concentrations (mmol/l) and daily milk yield on the test day nearest to date of CLA. Body weight changes from calving to CLA (wt change) were calculated by subtracting the body weight of the nearest date to CLA from the body weight at calving. Similarly, BCS changes (bcs change) were calculated by subtracting the body condition score of the nearest date to CLA from the body condition score at calving. Gains in wt change/bcs change were indicated by positive numbers whereas losses in wt change/bcs change were indicated by negative numbers.

4.3 Results

4.3.1 Endocrine Fertility Parameters

Descriptive Statistics

A total of seventy seven heifers were included in the analysis on endocrine fertility parameters from eighty eight heifers. Ten heifers calved before milk sample collection commenced and one heifer was culled during the study. Thirty nine heifers were in *ad libitum* group (Group A) and thirty eight heifers were in restricted group (Group R).

CLA and subsequent ovarian cyclicity was classified as follows for the study heifers:

A similar number of Group A (17 heifers) and Group R (10 heifers) had normal pregnane profiles ($P=0.112$). However, there was a trend for less Group A (3 heifers) than Group R (9 heifers) to have DOV1 classified profiles ($P = 0.053$). Meanwhile, Group A (11 heifers) and Group R (12 heifers) had DOV2 pregnane profiles with no group differences, ($P=0.746$). Then, Group A (5 heifers) and Group R (4 heifers) were classified as PCL1 with no significant differences between the 2 groups, ($P=0.754$). Finally, Group A (3 heifers) and Group R (3 heifers) had PCL2 pregnane profiles with no group differences, ($P=0.974$); Table 4.1, Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4 and Figure 4.5.

Table 4.1 Description and incidence of the pregnane profiles in Group A and Group R.

Type of profile	Definition	Group A n (%)	Group R n (%)	Total n (%)	P value
Normal	First rise in pregnane within 45 days postpartum, followed by regular cyclicity	17 (22.1)	10 (12.9)	27 (35.0)	0.112
DOV1	Low pregnane for ≥ 45 days postpartum	3 (3.9)	9 (11.7)	12 (15.6)	0.053
DOV2	Low pregnane for >12 days between adjacent luteal phases	11 (14.3)	12 (15.6)	23 (29.9)	0.746
PCL1	High pregnane for ≥ 19 days during the first cycle postpartum	5 (6.5)	4 (5.2)	9 (11.7)	0.754
PCL2	High pregnane for ≥ 19 days during the second or subsequent cycles	3 (3.9)	3 (3.9)	6 (7.8)	0.974
Total		39	38	77	

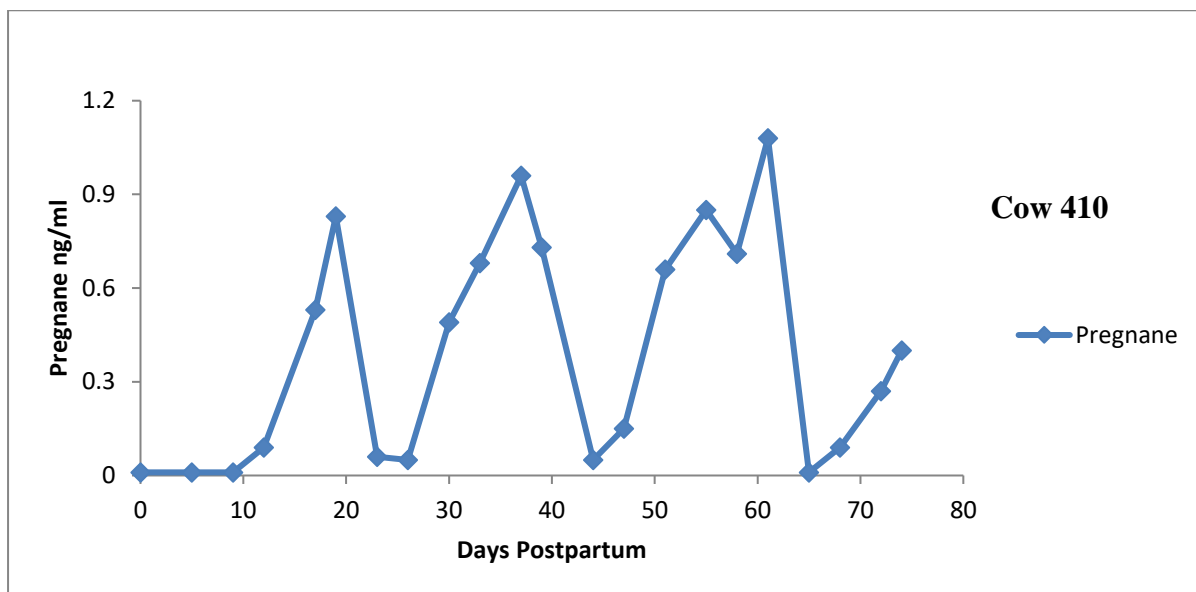


Figure 4.1 An example of a typical pregnane profile classified as normal commencement of luteal activity and subsequent cyclicity, (Cow 410).

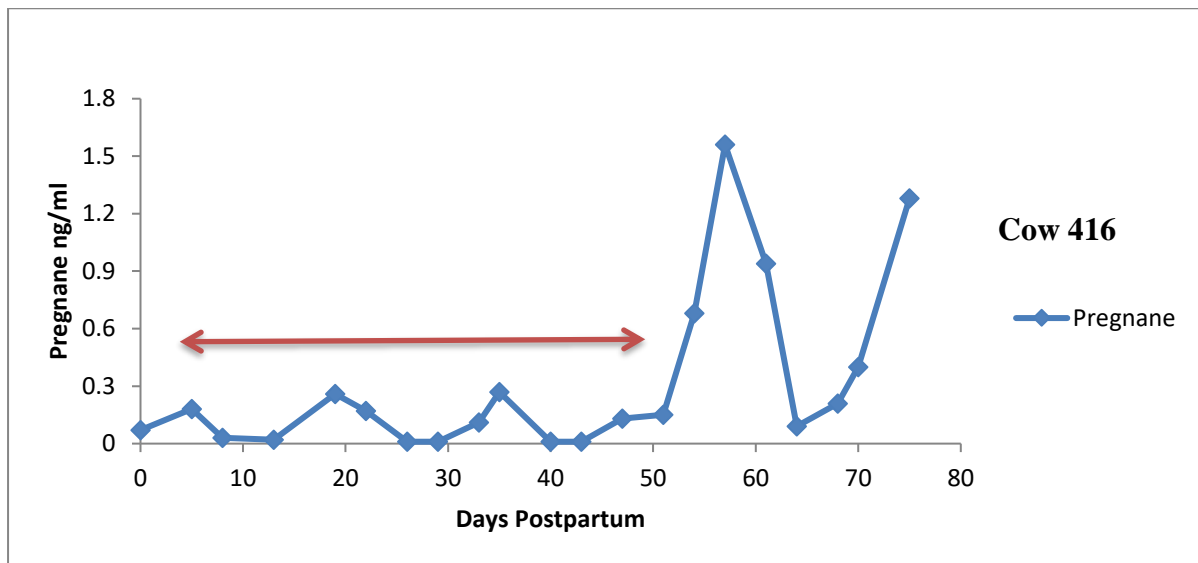


Figure 4.2 An example of a typical pregnane profile classified as delayed ovulation type I (DOV1), (Cow 416).

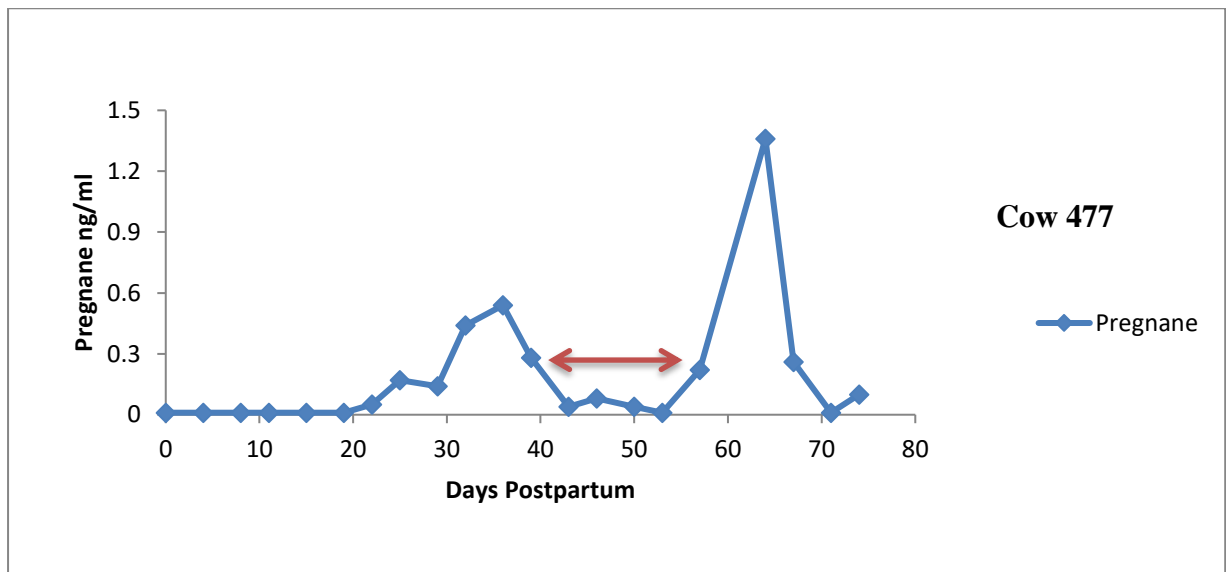


Figure 4.3 An example of a typical pregnane profile classified as delayed ovulation type II (DOV2), (Cow 477).

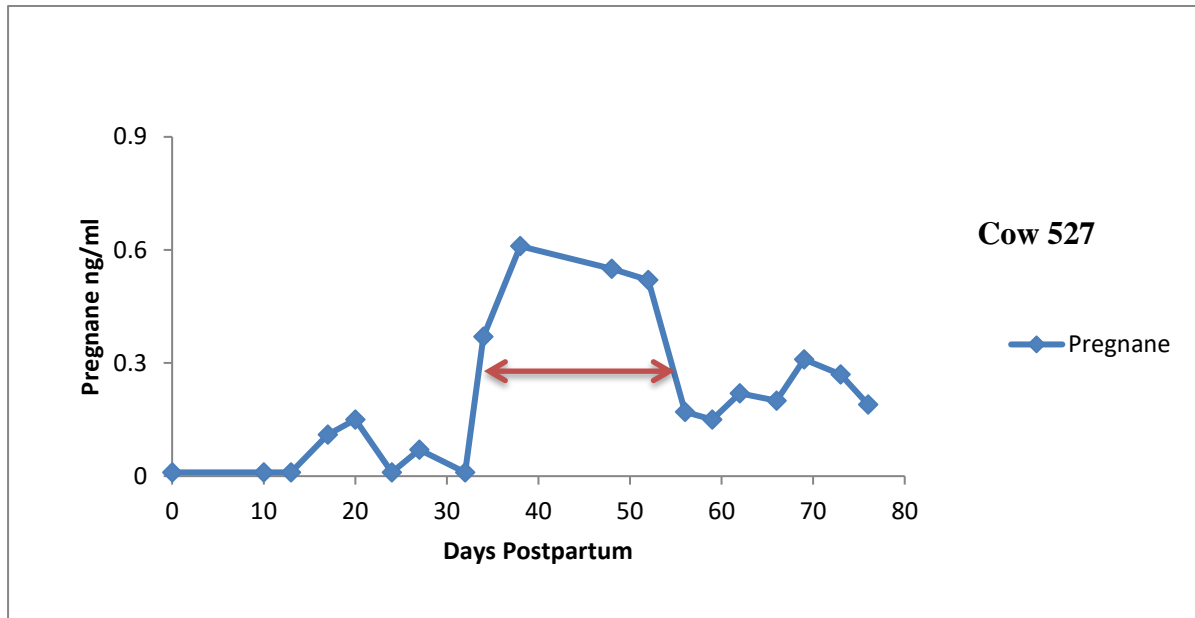


Figure 4.4 An example of a typical pregnane profile classified as persistent corpus luteum type I (PCL1), (Cow 527).

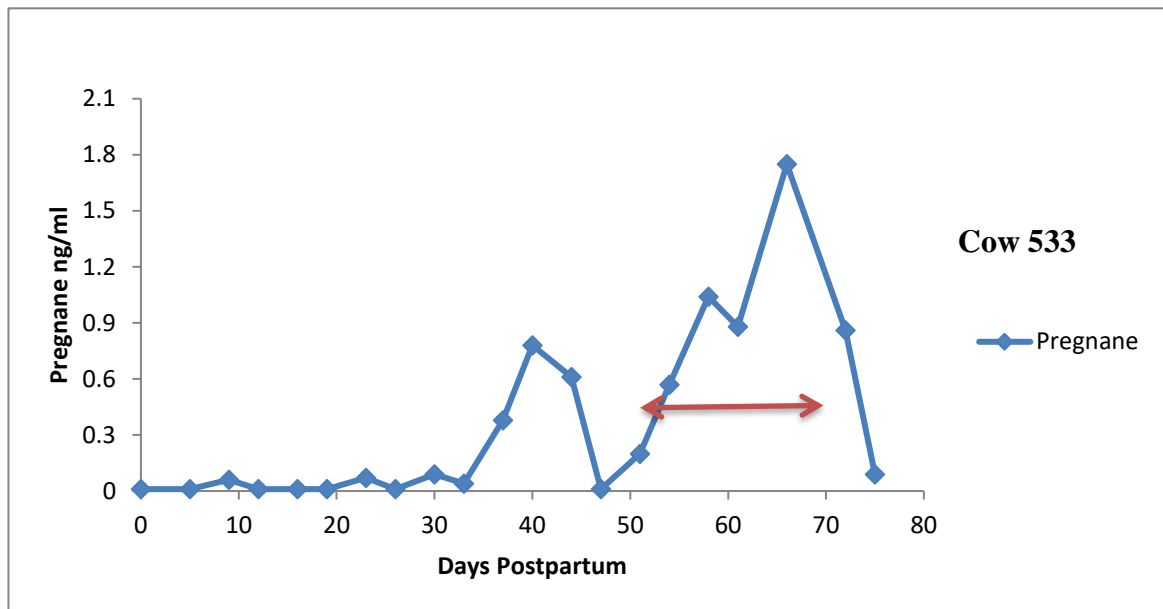


Figure 4.5 An example of a typical pregnane profile classified as persistent corpus luteum type II (PCL2), (Cow 533).

4.3.1.1 Commencement of Luteal Activity (CLA)

Whilst there were no significant differences in median time to CLA between the 2 groups, Group A: 23 days and Group R: 26 days, ($P=0.592$), appraisal of Figure 4.6 suggests that after approximately 25 days there was a slight trend for heifers in Group A to commence luteal activity earlier than their Group R counterparts. However, when data from 25 days onwards were compared the difference was not statistically significant ($P=0.183$, log-rank test).

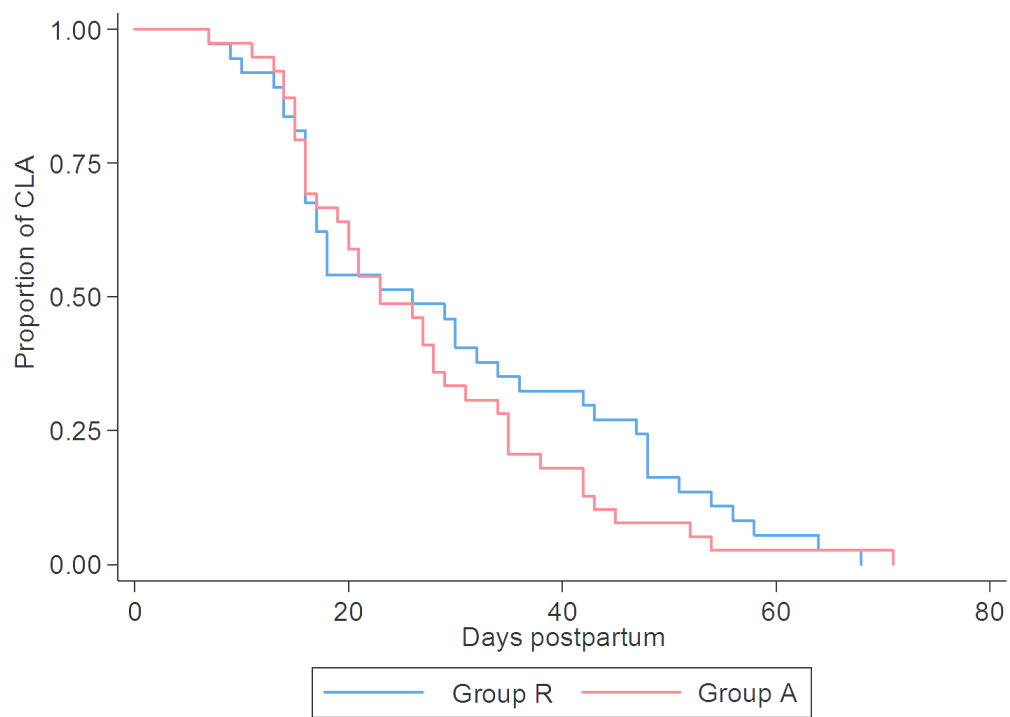


Figure 4.6 Kaplan-Meier survival curve for time to CLA for Group A (*ad libitum*) (n=39) and Group R (restricted) (n=38), (P=0.337, log-rank test).

4.3.1.2 *Time to CLA*

Univariate results from Cox proportional hazard models are presented in Table 4.2.

There were no significant associations at the $P < 0.050$ level although there was a non-significant ($P = 0.075$) association between a higher milk yield during the first week of lactation and a reduced hazard ratio for time to CLA (0.93 95% CI 0.86 – 1.01 per litre increase in yield). Similarly there was a non-significant ($P = 0.063$) association between increased β -hydroxybutyrate concentrations at time of CLA and a reduced hazard ratio (0.92 95% CI 0.85 – 1.00 for each 0.1 mmol/l increase).

Table 4.2 Univariable Cox regression model with time to CLA, hazard ratio, 95% confidence interval and p-value.

Explanatory variable: Time to CLA	Hazard Ratio	[95% Confidence Interval]		P-Value
Group (A vs R)	1.28	0.80	2.04	0.293
Average daily milk yield week 1	0.93	0.86	1.01	0.075
Average daily milk yield week 2	0.94	0.89	1.01	0.102
Average daily milk yield week 3	0.95	0.90	1.01	0.105
Average daily milk yield week 4	0.96	0.90	1.01	0.105
Average daily milk yield week 5	0.96	0.91	1.01	0.098
Average daily milk yield week 6	0.96	0.91	1.01	0.092
Average daily milk yield week 7	0.96	0.91	1.01	0.087
Average daily milk yield week 8	0.96	0.91	1.01	0.084
Change in body weight from calving to CLA (wt change)	1.00	0.99	1.01	0.667
Change in body condition score from calving to CLA (bcs change)	1.04	0.35	3.06	0.947
β -hydroxybutyrate concentration nearest to CLA	0.92	0.85	1.00	0.063
Daily milk yield nearest to CLA	0.94	0.91	0.96	0.000

4.3.1.3 Luteal Phase and Inter Luteal Phase

There were no differences between groups in the length of first luteal phase, length of first inter luteal interval, length of second luteal phase and length of second inter luteal interval (Table 4.3).

Table 4.3 Median and interquartile range (IQR) for the length of first and second luteal phase (LP) and first and second length of inter luteal interval (ILI) for Group A and Group R.

Endocrine Parameter	Group A (Days)		Group R (Days)		P-Value
	Median	IQR	Median	IQR	
First LP	10.5	9.0	8.5	9.5	0.232
First ILI	7.5	6.0	10.5	10.0	0.105
Second LP	13.5	8.0	11.5	9.0	0.988
Second ILI	7.5	3.0	7.5	8.0	0.451

4.3.1.4 Pregnane Profile

The mean duration of each type of pregnane profile: Normal, DOV1, DOV2, PCL1 and PCL2 was not differed between Groups; Table 4.4.

Table 4.4 Mean per type of profile for interval from calving to commencement of luteal activity (CLA) for Group A and Group R.

Pregnane Profile	Group A (n) (Days)	Group R (n) (Days)	P-Value
Normal	25.4 \pm 2.6 (n=17)	21.8 \pm 3.4 (n=10)	0.407
DOV1	59.3 \pm 6.0 (n=3)	56.0 \pm 2.6 (n=8)	0.572
DOV2	24.4 \pm 2.7 (n=11)	23.3 \pm 3.9 (n=12)	0.822
PCL1	23.1 \pm 5.9 (n=5)	30.0 \pm 6.9 (n=4)	0.467
PCL2	23.6 \pm 6.2 (n=3)	20.3 \pm 3.1 (n=3)	0.654

4.3.2 Traditional Fertility Parameters

There were no differences between groups in interval from calving to first oestrous, calving to first service, calving to conception, calving interval and the number of services required for conception (Table 4.5).

Table 4.5 Median and interquartile range (IQR) for the reproductive performance for Group A and Group R.

Reproductive Parameter	Group A		Group R		P-Value
	Median	IQR	Median	IQR	
Calving to first oestrous (days)	33.0	12.0	31.0	16.0	0.326
Calving to first service (days)	61.0	11.0	64.5	22.0	0.137
Calving to conception (days)	81.0	37.0	90.0	43.0	0.286
Calving interval (days)	364.0	42.0	376.0	54.5	0.603
Number of service (no.)	2.0	2.0	2.0	2.0	0.537

4.3.3 Health

No animals in either group required assistance during parturition. There was also no difference between Group A and Group R in incidence of retained foetal membrane (RFM) which occurred in three heifers where 2 heifers in the Group A and 1 heifer in the Group R, ($P=0.631$).

There was no difference between Group A and Group R in vaginal mucous score at postnatal check (PNC). Eighty nine per cent of heifers (Group A: 42 heifers; Group R: 35 heifers) had a score of 0 (clear), 1% (Group A: 1 heifer) a score of 1 (flecks of pus), 3% (Group R: 3 heifers) a score of 2 (mild endometritis) and 7% (Group A: 3 heifers; Group R: 3 heifers) a score of 3 (endometritis), ($P=0.410$).

There was no difference between Group A and Group R in incidence of lameness with 10 heifers in the Group A and 9 heifers in the Group R treated for lameness, ($P=0.981$).

Only two heifers (2.3%) were treated for mastitis, one in each group, thus not different between Groups ($P=0.935$). Five heifers (5.7%) were treated for other disease conditions with no group differences, ($P=0.326$).

4.4 Discussion

In the present study, 65% of heifers showed at least one atypical pregnane profile, whilst 35% of heifers showed normal pregnane profile in the initial 80 days of the first lactation. This figure is higher than the 42% reported by McCoy et al. (2006), 62% reported by Law et al. (2009c) and 62.2% reported by Gilmore et al. (2011). Royal et al. (2000) stated that the proportion of cows exhibiting at least one atypical ovarian hormone patterns had increased from 32% to 44% between 2 databases collected for the periods of 1975 to 1982 and 1995 to 1998, respectively. Therefore, our recent data are consistent with a continuation of this upward trend in occurrence of abnormal cyclicity postpartum.

4.4.1 *Commencement of Luteal Activity*

In the present study, there was no difference in time to CLA between Group A and Group R. To the best of our knowledge, the present study is the first to evaluate and compare CLA in heifers that received different milk replacer during pre-weaning period. CLA is an indicator of the interval from calving to first ovulation, which occurs on average four to five days before CLA (Darwash, Lamming and Woolliams, 1997a). The time to CLA recorded in the present study is in general agreement with other studies that previously reported by Darwash et al. (1997b), Royal et al. (2000), McCoy et al. (2006), Law et al. (2009c) and Gilmore et al. (2011). The results of the present study, support the suggestion of Royal et al. (2000), the interval to CLA had not changed in the last 20 years. Increases in time to CLA may reflect the inability of the ovulatory follicle to produce sufficient oestradiol to stimulate an LH surge and ovulation in the early postpartum period and may be related to the degree of negative daily energy balance suppressing LH pulse frequency. Nutritional factors and their effect on energy balance in early lactation (Beam and Butler, 1997; Opsomer et al., 2000) and

periparturient disease (Opsomer et al., 2000) appear to be the main factors influencing onset of cyclicity or luteal activity and different feeding strategy during early life of heifer calves in this study did not appear to change the effect of these factors on CLA.

All the animals in the current study were heifers. Garmo et al. (2009) reported that first parity heifers had longer intervals to CLA than second parity cows. This statement agrees with Petersson et al. (2006b), but contrasts with the reports from Darwash et al. (1997a) who stated that the interval increase with parity.

From our study, there was no effect of milk yield on CLA. The reason for this because the dataset was relatively small, thus decreasing the likelihood of detecting a significant association. Windig et al. (2008) could not find an association between milk production and luteal activity in their study. However, Garmo et al. (2009) stated that cows selected exclusively for high milk yield experienced a longer interval from calving to CLA than cows with no selection response for milk yield. The interval from calving to CLA in cows selected for both milk production and fertility was intermediate between high and low milk production lines.

In the present study, changes in both body weight and body condition score did not affect CLA. There were only small losses or gains in body weight and BCS during early postpartum period and also first parity animals are still growing. Buckley et al. (2003) reported that a significant effect of body weight at the start of the herd breeding season and body weight change from the start of breeding to 90 days thereafter on pregnancy rate at first service, suggesting that body weight is potentially an important determinant of the likelihood of reproductive success. In our study, change in body weight during early breeding of the heifers was very minimal therefore, the effect not so obvious. Roche et al. (2007) stated that BCS at calving was greater in first parity animals, decreased in second parity and progressively

increased with parity. However, above studies did not mention effect of changes in both body weight and body condition score on CLA.

In our study, β -hydroxybutyrate (BHB) concentration also did not influence CLA. This is because peak BHB concentration occurred after CLA (Chapter 3). Cows with higher plasma concentrations of insulin-like growth factor 1 (IGF-1) had an increased likelihood of a shorter interval to CLA, whereas plasma concentrations of BHB, insulin, glucose and non-esterified fatty acids (NEFA) were not associated with CLA (Patton et al., 2007). Meanwhile, higher concentrations of BHB and growth hormone; and lower concentrations of insulin, glucose and IGF-1 in cows with high genetic merit for milk yield as reported by Gutierrez et al. (2006).

4.4.2 Pregnane Profiles

In our study, the incidences of DOV2, PCL1 and PCL2 were not different between Group A and Group R heifers. To the best of our knowledge, the present study is the first to evaluate and compare pregnane profiles in heifers that received different milk replacer feeding protocols during pre-weaning period. In this study, there was a trend for Group R heifers to have high incidence of DOV1 classified profiles compared to Group A. It is postulated that influence of epigenetic to be involved. Epigenetic effects may be triggered by conditions associated with natural biological process or by adverse conditions such as negative energy balance, heat stress, exposure to toxins or other disturbances. Many responses of dairy cattle to environmental effects are regulated by epigenetic or closely-related processes at the cellular level in animals. Even though the influence of epigenetic mechanisms was not studied, the outcome of such event has been observed (Robison et al., 1988; DeNise et al., 1989).

In the present study, the incidence of DOV1 (15.6%) was within the range of values reported by other studies (11% by Lamming and Darwash, 1998; 12.9% by Royal et al., 2000; 17.4% by McCoy et al., 2006; 12% by Garnsworthy et al., 2009; 20.7% by Law et al., 2009c and 12.5% by Gilmore et al., 2011).

The incidence of DOV2 (29.9%) was higher compared to previously reported values by Lamming and Darwash (1998) of 13%, 16% by Royal et al. (2000), 13% by Garnsworthy et al. (2009), 28% by Law et al. (2009c) and 16.3% by Gilmore et al. (2011).

In the current study, there appears to have been a substantial increase in incidence of all abnormal pregnane profile types, particularly delayed ovulation type (DOV1 and DOV2). This condition might be related to nutrition during early postpartum. Cows with DOV1 and DOV2 profiles had lower 100 days milk protein concentrations and lower body condition scores in the first 100 days of lactation in one study, suggesting an association with excessive negative energy balance in early lactation (McCoy et al., 2006). Delayed ovulation may be caused by a delay in, or failure of the LH surge, that should stimulate ovulation. It has been demonstrated that declining of follicle stimulating hormone (FSH) and increasing LH are associated with the differentiation and maturation of dominant follicles (Webb et al., 2004, 2007); thereby increasing the chance of ovulation in response to a LH surge (Beam & Butler, 1998, 1999). Differentiation and maturation of dominant follicles are controlled by local ovarian growth factors that affect their response to LH (Webb et al., 2007). According to Peter et al. (2009), DOV2 may occur due to the insensitivity of the hypothalamus to positive feedback by oestradiol or to altered follicular responsiveness to gonadotropic support, mediated via metabolic hormones such as IGF-1 and insulin (Beam & Butler, 1999). DOV2 also may be caused by incomplete luteolysis of the corpus luteum from the previous cycle or a continuing luteinised ovarian structure is present (Lee et al., 1985; Sirois & Fortune, 1990; Sartori et al., 2004).

The incidence of PCL1 in the present study was 11.7%, which is low than previously published work, with values of 16%, 19%, 19% and 16.4% from Taylor et al. (2003), McCoy et al. (2006), Law et al. (2009c) and Gilmore et al. (2011), respectively.

The incidence of PCL2 was 7.8%, which is comparable to values of 6.4% (Lamming & Darwash, 1998) but lower than that observed in a range of more recent studies (11% (Taylor et al., 2003), 11.9% (McCoy et al., 2006), 15% (Garnsworthy et al., 2009), 20.7% (Law et al., 2009c) and 34.4% (Gilmore et al., 2011)).

In the present study, the low occurrence of PCL could be explained by the low number of postpartum problems that happen in the heifers. Opsomer et al. (2000) stated that the most important risks for delayed luteolysis were early CLA, occurrence of metritis, retained placenta, calving problems and abnormal vaginal discharge. Petersson et al. (2006) also reported that endometritis to be a risk factor for PCL. Therefore, prolonged luteal phase could be the result of uterine problems rather than ovarian pathology.

In previous studies and also this study, the occurrence of a persistent corpus luteum was associated with a longer calving interval. An extended luteal phase can occur in 2 conditions; (i) when there is delayed luteolysis in the absence of pregnancy and (iii) when an oestradiol producing dominant follicle is absent at the time of luteal regression (Peter et al., 2009). Delayed luteolysis in the absence of pregnancy can also be caused by a suboptimal uterine environment (presence of bacterial pathogens) that can disturb the normal hormonal and luteolytic mechanisms (Sheldon et al., 2006). However, there was very low incidence of endometritis in our study therefore, the effect of suboptimal uterine environment in the heifers not so obvious.

4.4.3 Luteal Phase

In our study, the lengths of first and second luteal phase were not different between Group A and Group R heifers. To the best of our knowledge, the present study is the first to evaluate and compare length of luteal phase in heifers that received different milk replacer feeding regimes during pre-weaning period.

In the present study, the mean length of the first luteal phase (10.8 ± 0.7 days) was similar to that reported in other studies, whereas Garmo et al. (2009) stated the length of first luteal phase to be 10.5 days in Norwegian Red cows and Royal et al. (2000) reported that the length of first luteal phase to be 10.8 days in British Friesian from 1975 to 1982 and the same study also reported that the length of first luteal phase to be 14.6 days from 1995 to 1998 after there had been a large breed substitution of British Friesian with North American Holstein. However, Royal et al. (2000) recorded that the length of the second luteal phase was 13.2 days and 15.1 days from 1975 to 1982 and from 1995 to 1998, respectively; was higher compared to our study (12.4 ± 0.9 days). Therefore, measurement of luteal phase length may provide an indication of uterine environment of the cow.

4.4.4 Inter Luteal Interval

In our study, lengths of first and second inter luteal interval were not different between Group A and Group R heifers. To the best of our knowledge, the present study is the first to evaluate and compare length of inter luteal interval in heifers that received different milk replacer feeding protocols during pre-weaning period.

The mean length of first inter luteal interval (10.9 ± 0.8 days) was longer compared to that 8.1 days and 7.7 days from 1975 to 1982 and from 1995 to 1998, respectively reported by

Royal et al. (2000). However, Royal et al. (2000) recorded that the length of the second ILI was 7.9 days and 7.5 days from 1975 to 1982 and from 1995 to 1998, respectively; was similar to our study (8.0 ± 0.6 days). Thus, measurement of ILI allows indirect detection of delayed ovulation, delayed luteinisation and the presence of an incompetent corpus luteum.

4.4.5 Reproductive Parameters

In our study, all the reproductive parameters (calving to first oestrous, calving to first service, calving to conception, calving interval and number of service) were not different between Group A and Group R heifers, suggesting that pre-weaning nutrition had no impact on overall reproductive performance. To the best of our knowledge, the present study is the first to evaluate and compare reproductive parameters in heifers that received different milk replacer feeding regimes during pre-weaning period.

In the current study, the mean length of calving to first service (66.1 ± 1.6 days) was shorter compared to other studies for example, Dhaliwal et al. (1996) reported that (low yielding cows: 75.8 days; high yielding cows: 80.3 days) and Darwash et al. (1997b) (71.2 days). Similarly, Mayne et al. (2002) stated that the calving to first service was 84.3 days, with a range from 67 to 119 days between the herds in their study. However, the target for calving to first service for the UK national dairy herd is 73 days (Hanks & Kossaibati, 2014). The differences between the levels of production in the herds indicated that the farmers to operate different management practices; with some targeting a 365-day calving interval, some targeting higher milk production and advancing or delaying the earliest date of insemination accordingly (Mayne et al., 2002).

The mean calving to conception interval (102 ± 6.6 days) also was shorter compared to Dhaliwal et al. (1996) reported that (low yield: 99.8 days; high yield: 118.8 days). However, Esslemont and Peeler (1993) estimated that the optimum interval for calving to conception to be 85 days.

In the present study, the mean length of calving interval (383.2 ± 6.6 days), which is in the middle when compared with previously published work, with values of 370.0 days and 390.4 days, from Bulman (1977) and Royal et al. (2000), respectively. Mayne et al. (2002) reported that the calving interval was 407.2 days in their study, which was higher compared to other studies. Calving interval reported in the literature range from 368 days (Evans et al., 2006) to 398 days (Haile-Mariam et al., 2003), but recent study from the Scottish Agriculture College's Langhill herd found calving interval to be 403 days (Pollock and Coffey, 2008). In general, herds with shorter calving intervals are associated with higher heat detection rates and a shorter interval to first AI service. According to Mayne et al. (2002), the cows in their study had lower body condition scores in the dry period before they calved and also lost less body condition in the post calving period, in association with a smaller negative energy balance in early lactation. Therefore, to achieve an average calving interval of less than 365 days, this is achievable by minimising the cows' negative energy balance in early lactation, together with good heat detection, early insemination of cows after calving and a good AI technique.

4.4.6 Health

In our study, there was no difference in incidence of any of the measured health parameters (calving ease, RFM, endometritis, lameness, mastitis and disease incidence) between Group A and Group R heifers. To the best of our knowledge, the present study is the first to evaluate

and compare health parameters in heifers that received different milk replacer feeding protocols during pre-weaning period. However, we do understand that our dataset was relatively small with a low incidence of disease occurring, thus decreasing the likelihood of detecting any significant associations.

Incidence of RFM in the present study was about 4%, and the average incidence of RFM in the United Kingdom was estimated to be between 4 and 8% (Laven & Peters, 1996). Average incidence of RFM ranges from 4 to 11% of calving (Eiler, 1997). Many cows with RFM have elevated body temperature as a sign of acute metritis. There was a pattern of lower values for first-service conception rates, total conception rates and proportion of cows pregnant by 200 days postpartum (Drillich et al., 2006).

Based on vaginal mucous score, the incidence of endometritis in the present study (10%) was lower than the incidence of 40% and 31% reported by Garnsworthy et al. (2009) and Williams et al. (2005), respectively. The prevalence of subclinical endometritis is quite variable among herds. About 35% of dairy cows may be clinically infected in the first 21 days postpartum (metritis) and about 10-20% will remain infected or develop endometritis (Borsberry & Dobson, 1989; Sheldon et al., 2009). The proportion of infected animals or uterine bacterial amount usually increases between the 7th and 14th day postpartum. This observation leads to the assumption that there is not only a bacterial contamination during postpartum that is responsible for uterine problems (Sheldon, 2004).

Overall incidence of lameness was approximately 22%. Lameness was identified as the third most costly disease of dairy cattle, with mastitis and poor fertility being the most costly (Kossaibati & Esslemont, 1997). It has been found to affect the economic performance of dairy cattle in several ways: loss of body weight, reduced milk yield, poor fertility, treatment costs and premature culling (Kossaibati & Esslemont, 1997). Culling due to lameness was

3.5% in the first lactation and rose to 9% in the seventh lactation (Kossaibati & Esslemont, 1997). However, other studies in the United Kingdom have reported that differently for different type of farming systems; for example 24% for organic herds (Huxley et al., 2004), 15% for grazing herds and 39% for zero-grazing herds (Haskell et al., 2006) and 36.8% reported by Barker et al. (2010).

Other disease incidence was recorded about 6% in our study and there was no different between Group A and Group R heifers. It is not surprising that the heifer calves that received lower volumes of milk replacer during early life did not have an increased likelihood of developing disease during the study. They may have been able to compensate with the condition where their immunity was not compromised.

4.5 Conclusion

The different milk replacer feeding strategies that were applied during the early life of heifer calves in the present study did not have a statistically significant effect on the fertility (either endocrine or reproductive) and health performance of the dairy heifers. However, there was a trend for Group R heifers to have high incidence of DOV1 classified profiles compared to their counterpart.

Present study fits the trend for the incidence of abnormal pregnane profiles may be increasing over time. Increased interval to CLA and abnormal pregnane profiles were associated with reduced fertility performance manifested in an increased interval to first AI service and finally, prolonged calving intervals.

Chapter 5

Effect of Different Pre-Weaning Feeding Strategies in Early Life of Heifer Calves on First Lactation Milk Yield

5.1 Introduction

Nutrient intake in the early stage of life may have long term effects on milk production. Milk yield is one of the most important factors for sustainability of dairy cattle production. It is affected by many phenotypic and genotypic factors for example: age, breed, lactation number and period, properties of teat and udder of cow, management and animal nutrition (Koc, 2006).

The practice of feeding high energy diets for fast growth rates enables heifers to achieve breeding size and puberty earlier, potentially decreasing age at first calving and costs associated with raising replacement heifers (Raeth-Knight et al., 2009). This practice, although known to increase rates of skeletal and body weight growth, possibly reduces development of the mammary ductal epithelium (Sejrsen and Purup, 1997). Thus, mammary secretory epithelium (alveoli), which develops during gestation, depends on ducts that have developed in early life (Sejrsen et al., 2000). Therefore, a main objective in raising replacement heifers is to manage them for rapid structural growth along with optimal development of the mammary gland.

In heifers, allometric mammary growth occurs when the udder grows at a faster rate than the rest of the body. Period from birth to 3 months of age is the first developmental stage of the mammary gland, during which mammary gland grows at an allometric rate until around the onset of puberty and then becomes isometric relative to overall body growth (Sinha and Tucker, 1969). Allometric growth of the mammary fat pad and of the mammary ducts begins between 2 and 3 months of age (approximately 100 kg of body weight) (Sinha and Tucker, 1969). For example, in an experiment designed to evaluate the impact of greater energy and protein intake during 2 periods of early mammary development in heifers (2 to 8 weeks of

age and 8 to 14 weeks of age), Brown et al. (2005) concluded that the feeding amount did not significantly affect the percentage of epithelial tissue in mammary parenchyma.

From 3 months of age until completion of several oestrous cycles, relative growth rates of mammary gland are more than 3-fold greater than body weight. This period is considered as the allometric phase of mammary development (Sinha and Tucker, 1969; Swanson and Poffenbarger, 1977). Once a heifer reaches puberty between 9 and 11 months of age (250 to 280 kg of body weight), the mammary gland returns to an isometric rate of growth. After that, there seems to be no effect of feeding level on mammary development (Sejrsen et al., 1982). Many studies on the effects of body and mammary gland growth rates on milk production were conducted during the allometric growth phase, a period which mammary ductile tissue is developing within the mammary fat pad (Sejrsen et al., 1998). However, the impact of nutrition on pre-pubertal mammary growth and future milk production remains controversial (Meyer et al., 2006).

Many studies comparing the effects of suckling, controlled intakes and *ad libitum* feeding of calves from birth up to 56 days of life have shown that increased nutrient intake before 56 days of life from milk resulted in increased milk yield ranging from 450 to 1,300 kg during the first lactation (Bar-Peled et al., 1997; Shamay et al., 2005; Terre et al., 2009; Moallem et al., 2010; Soberon et al., 2012). However, other studies using different amounts of milk replacer during the pre-weaning period showed no significant effect on first lactation milk yield (Morrison et al., 2009; Raeth-Knight et al., 2009). In a recent study by Moallem et al. (2010), the effects of pre-weaning nutrition on first lactation milk yield were related to the type and quality of nutrients fed to the calves. They reported that 10.3% higher milk yields during first lactation from heifer calves fed whole milk *ad libitum* compared to heifer calves fed milk replacer *ad libitum* during the same period and suggested that milk replacer did not

contain the same biologically active factors as milk and therefore, did not impart any lactocrine effects on the calves.

Several lactation models have been proposed to predict milk production or milk components. Model accuracy and precision vary because of the effect of management, genetics and environment. Lactation models developed include, nonlinear MilkBot model (Ehrlich, 2011), best prediction (BP) model (Cole and VanRaden, 2006) and a null model (NM) based on a stepwise function (Tedeschi, 2006). MilkBot (MB) model was developed by Ehrlich (2011). It is a nonlinear lactation model designed to predict daily milk yields using a lactation curve model that is flexible to accommodate disease and managements effects. A study conducted by Hostens et al. (2012) investigated the relations between metabolic diseases and milk production. From raw milk production data, no statistically significant relations were found. However, using MilkBot for modelling of individual lactations allowed the demonstration of highly significant associations between diseases and production. BP is a method in which test day data are compared with breed and parity specific herd lactation curves. However, Cole and VanRaden (2006) stated that BP systematically underestimates daily yield in early and late lactation and overestimates daily yields in the middle of lactation.

The aims of the present study were to determine the effect of milk replacer feeding strategy in early life of heifer calves on their first lactation performance by comparing milk parameters between *ad libitum* milk replacer fed calves (Group A) and restricted milk replacer fed calves (Group R) groups by using the MilkBot lactation model.

5.2 Materials and Methods

5.2.1 Animals

The present study was conducted from 4th April 2013 until 16th April 2015 on 88 pedigree Holstein heifers at Wood Park Farm, University of Liverpool, United Kingdom. Heifers were between 23-32 months of age. The farm milked approximately 170 Holstein Friesian cows with an annual lactation yield about 10,500 litres on a three times daily milking regime. All cows were housed year round apart from during the last 100 days of lactation during which they were allowed out onto grazing during the summer months. All non-lactating pregnant (dry) cows were housed throughout the eight week dry period. The calving pattern on the farm was described as “all year round” with no seasonal trends. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 under a UK Home Office licence for work on living animals and with the approval of the University of Liverpool Ethical Review Process.

5.2.2 MilkBot Lactation Model

The MilkBot lactation model (Ehrlich, 2011), was used to summarize the magnitude and shape of each individual lactation curve. It can be expressed functionally as

$$Y(t) = a * e^{-dt} (1 - 0.5 * e^{(c-t)/b})$$

where $Y(t)$ is total daily milk production on day t of the lactation, e is *Euler's number* (approximately 2.71828) and parameters a , b , c and d control the shape of the curve. Milk weights data on the day of sampling and monthly were exported from Excel and sent to Dr Ehrlich to fit to the MilkBot model using a proprietary maximum likelihood fitting algorithm (DairySight LLC, Argyle, New York). The fitted parameter values generated by this process

quantify magnitude and selected aspects of the shape of each curve. Specifically, lactation scale is measured by the a parameter in the MilkBot function. It is a simple linear scalar with equal influence at all stages of lactation. The parameter b , is called the ramp parameter; measures the steepness of the postparturient increase in production, therefore, it is most influenced by changes in early lactation. Higher ramp values correspond to a slower increase in production. The MilkBot offset parameter, c , is the theoretical offset between parturition and the physiological start of lactation. The decay parameter, d , relates to senescence and loss of productive capacity and is influenced by cumulative changes in productive capacity occurring throughout the lactation. Decay can be expressed as half-life, called persistence, corresponding approximately to the time in days for production to decrease by half in late lactation (Ehrlich, 2011). This methodology allows scale, ramp and persistence of individual lactations to be treated as independent variables in statistically models, along with the derived variables cumulative 305-d milk yield (M305), time to peak milk (Peak Day) and peak milk (Peak Milk); which are easily calculated directly from MilkBot parameters values (Ehrlich, 2011). Graphs of estimated milk yield in the first lactation against days in milk (DIM) for Group A (*ad libitum*) and Group R (restricted) were plotted based on the MilkBot model.

5.2.3 Statistical Analysis

All data were analysed using Stata 13 (StataCorp) with data expressed as mean \pm SEM or proportions where appropriate. Data were compared between Group A and Group R. They were checked for normality using normal quantile-quantile plots and basic descriptive statistics were carried out. Normally distributed data is described using mean values and standard errors of the mean. However, where data are not normally distributed, medians and inter quartile range (IQR) are quoted.

Data on total lactation of milk yield, 305 day milk yield (both actual and estimated using MilkBot), milk fat percentage and milk protein percentage were analysed and compared between the 2 groups using two-sample t-test. Individual animal mean SCC at the monthly over the whole lactation period was analysed using Wilcoxon rank-sum test. Differences were considered statistically significant if P values were equal or less than 0.05.

Simple correlation coefficients were determined to examine relationships among the following recorded variables: body weight at calving, age at calving, heart girth at calving, belly girth at calving, total lactation of milk yield, milk fat percentage, milk protein percentage and SCC.

A multivariable random effects linear regression model was fitted with both actual and MilkBot predicted 305 day milk yield in first lactation as the outcome variable. Explanatory variables offered to the initial model were: firstly calf associated variables namely calf sex and birth weight; secondly heifer associated variables namely pre-weaning dietary group (*ad libitum* milk replacer versus restricted milk replacer), age at calving, body weight at calving, BCS at calving, BHB value at calving and rumen fill score at calving. A backward stepwise method was used for selection of variables for inclusion in the final model taking $P < 0.200$ (likelihood ratio test) for retention of a variable. The variable “pre-weaning dietary group” was forced into the model as the outcome variable of primary interest. Heifer sire was included as a random effect.

5.3 Results

Descriptive Statistics

Lactational curves calculated from the MilkBot model parameter estimates are shown in Figure 5.1.

There were no significant differences between groups with respect to actual total lactational milk yield (Group A: 10162.6 ± 520.1 kg *versus* Group R: 9877.4 ± 483.1 kg), ($P=0.695$); actual 305 day milk yield (Group A: 9262.9 ± 306.2 kg *versus* Group R: 9371.6 ± 396.8 kg), ($P=0.826$) and MilkBot predicted 305 day milk yield (Group A: 9569.3 ± 240.4 kg *versus* Group R: 9669.0 ± 306.2 kg), ($P=0.796$).

There was a significant difference with respect to actual 305 day milk yield for heifers that gave birth to bull calves compared to heifer calves (bull calves: 9918.1 ± 316.2 kg *versus* heifer calves: 8793.8 ± 345.2 kg), ($P=0.020$). However, there was no significant difference to MilkBot predicted 305 day milk yield for heifers that gave birth to bull calves compared with heifer calves (bull calves: 9946.6 ± 269.6 kg *versus* heifer calves: 9329.9 ± 260.3 kg), ($P=0.105$).

There was a significant difference between birth weight of bull calves compared to heifer calves (bull calves: 44.0 ± 1.2 kg *versus* heifer calves: 39.9 ± 0.7 kg), ($P=0.002$).

Whilst there was no significant difference over the lactation period in mean monthly milk fat percentage (Group A: 3.99 ± 0.06 % *versus* Group R: 3.98 ± 0.07 %), ($P=0.856$) (Figure 5.2) and mean monthly milk protein percentage was significant higher in Group A compared to Group R (3.21 ± 0.03 % *versus* 3.14 ± 0.03 % respectively), ($P=0.048$) (Figure 5.3).

There were no significant differences between groups with respect to total lactation period protein yield (Group A: 330.7 ± 15.3 kg *versus* Group R: 310.3 ± 13.8 kg), ($P=0.328$) and lactation fat yield (Group A: 407.6 ± 17.5 kg *versus* Group R: 390.5 ± 16.8 kg), ($P=0.485$).

5.3.1 Somatic Cell Counts (SCC)

The median somatic cell count for all monthly recordings for Group A was (67.5×10^3 cells/ml) [interquartile range (IQR), 28.0 to 107.0×10^3 cells/ml] and Group R was (35.0×10^3 cells/ml) IQR (25.0 to 58.0×10^3 cells/ml). There was a significant difference between the 2 groups, ($P=0.035$) (Figure 5.4).

5.3.2 MilkBot Parameters

Scale

There was no significant difference in the estimated scale parameter a between Group A and Group R heifers. The median of scale for Group A was (38.3 kg) [interquartile range (IQR): 37.9 to 39.7 kg] and Group R was (38.3 kg) (IQR: 38.3 to 48.4 kg), ($P=0.449$).

Ramp

There was a significant difference in the estimated ramp parameter b between the 2 groups (Group A median (29 days) IQR (25 to 32 days) *versus* Group R median (32 days) IQR (29 to 34 days), ($P=0.026$).

Decay

There was no significant difference in the estimated decay parameter d between Group A and Group R. The median of decay for Group A was (0.001 per day) IQR (0.0008 to 0.0016 per day) and Group R was (0.001 per day) IQR (0.0007 to 0.0016 per day), (P=0.962).

Persistence

There was no significant difference in the estimated persistence parameter between Group A and Group R. The median of persistence for Group A was (620 days) IQR (442 to 871 days) and Group R was (620 days) IQR (430 to 996 days), (P=0.955).

Peak Milk

There was no significant difference in the estimated peak milk between the 2 groups (Group A median (34.4 kg) IQR (31.7 to 36.8 kg) *versus* Group R median (35.7 kg) IQR (31.6 to 40.4 kg), (P=0.249).

Peak Day

There was no significant difference in the estimated peak day between Group A and Group R heifers. The median of peak day for Group A was (78 days) IQR (69 to 88 days) and Group R was (81 days) IQR (74 to 89 days), (P=0.286).

5.3.3 Relationships among Variables

Actual total lactation milk yield showed a significant positive correlation with body weight at calving ($r=0.37$; $P=0.001$), heart girth at calving ($r=0.31$; $P=0.005$) and belly girth at calving ($r=0.34$; $P=0.002$). However, total lactation milk yield was not significantly correlated with β -hydroxybutyrate (BHB) values at calving ($r=0.11$; $P=0.350$), age at calving ($r=0.20$; $P=0.076$) and body condition score at calving ($r=0.21$; $P=0.062$).

Somatic cell counts showed a significant positive correlation with age at first calving ($r=0.30$; $P=0.009$) and milk fat percentage ($r=0.21$; $P=0.040$), respectively. However, SCC showed a significant negative correlation with total lactation milk yield ($r=-0.12$; $P=0.020$).

5.3.4 Actual 305-d milk yield and MilkBot predicted 305-d milk yield

The following variables: pre-weaning dietary group (*ad libitum* milk replacer versus restricted milk replacer), body weight at calving, age at calving, calf sex and calf birth weight remained in the final regression model for actual 305-d milk yield (Table 5.1) and also for MilkBot predicted 305-d milk yield (Table 5.2). Bull identity accounted for 8.8% (95% CI 0.5 – 63.2%) for actual 305-d milk yield and 10.2% (95% CI 1.2 – 51.9%) for MilkBot predicted 305-d milk yield of the residual error. Pre-weaning dietary group was retained in the final model since it was the primary explanatory variable of interest.

Heifer associated variables: there were significant association between body weight at calving with actual 305-d milk yield (13.5 95% CI 5.3 – 21.8 per kg increase in body weight), ($P=0.001$) and also with MilkBot predicted 305-d milk yield (11.4 95% CI 5.1 – 17.8 per kg increase in body weight), ($P<0.050$). However, there were no significant association between age at calving with actual 305-d milk yield ($P=0.183$) and also with MilkBot predicted 305-d

milk yield ($P=0.203$). Pre-weaning dietary group was not significantly associated with either actual, ($P=0.526$) or MilkBot predicted, ($P=0.737$) 305-d milk yield.

Calf associated variables: calf sex and calf birth weight.

There was a significant association between calf sex and actual 305-d milk yield (-1052.4 95% CI -1919.0 - -185.7 reduction in kg milk yield in female calf), ($P=0.017$). However, there was no significant association between calf sex and MilkBot predicted 305-d milk yield ($P=0.183$).

There was a trend in the association between calf birth weight and actual 305-d milk yield ($P=0.056$). However, there was a significant association between calf birth weight and MilkBot predicted 305-d milk yield ($P=0.019$).

Table 5.1 Multivariable regression model for association between actual 305-d milk yield and pre-weaning dietary group, body weight at calving, age at calving, calf sex and birth weight; with bull identity included as a random effect.

Outcome Variable: Actual 305-d Milk Yield (kg)	Coefficient	[95% Confidence Interval]		P Value
Pre-weaning dietary group (A vs R)	262.24	-547.57	1072.05	0.526
Body weight at calving (kg)	13.53	5.25	21.81	0.001
Age at calving (days)	-4.43	-10.94	2.09	0.183
Calf sex	-1052.36	-1919.03	-185.69	0.017
Birth weight (kg)	78.31	-1.87	158.49	0.056
Constant	2124.24	-3593.78	7842.26	0.467

Random-effects Parameters	Estimate	[95% Confidence Interval]	
Bull identity	261508.8	17347.66	3942138
Residual error	2714995	1858091	3967081

Residual Intraclass Correlation (ICC)

Level	ICC	[95% Confidence Interval]	
Bull	0.088	0.005	0.632

Table 5.2 Multivariable regression model for association between MilkBot predicted 305-d milk yield and pre-weaning dietary group, body weight at calving, age at calving, calf sex and birth weight; with bull identity included as a random effect.

Outcome Variable: MilkBot Predicted 305-d Milk Yield (kg)	Coefficient	[95% Confidence Interval]		P Value
Pre-weaning dietary group (A vs R)	106.36	-514.36	727.08	0.737
Body weight at calving (kg)	11.41	5.05	17.78	0.000
Age at calving (days)	-3.24	-8.23	1.75	0.203
Calf sex	-451.18	-1114.76	212.39	0.183
Birth weight (kg)	73.58	12.10	135.05	0.019
Constant	2657.23	-1728.01	7042.46	0.235

Random-effects Parameters	Estimate	[95% Confidence Interval]	
Bull identity	178824.9	21531.99	1485155
Residual error	1580168	1093635	2283148

Residual Intraclass Correlation (ICC)

Level	ICC	[95% Confidence Interval]	
Bull	0.102	0.012	0.519

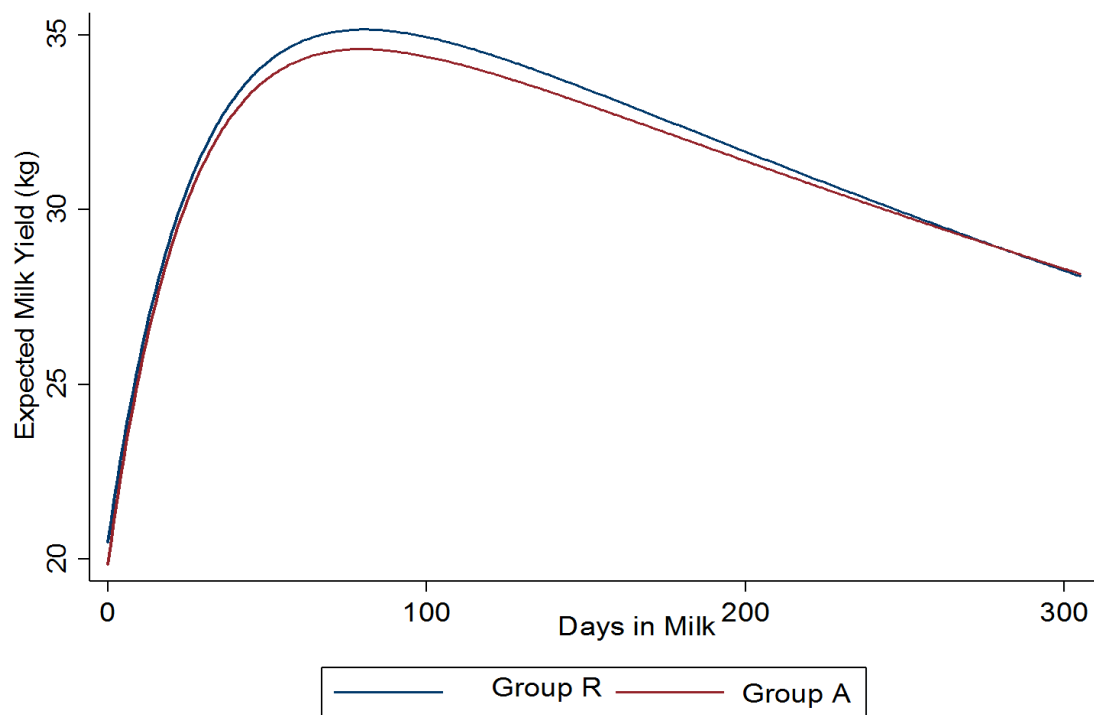


Figure 5.1 Graph for expected milk yield in the first lactation for Group A (*ad libitum*) and Group R (restricted).

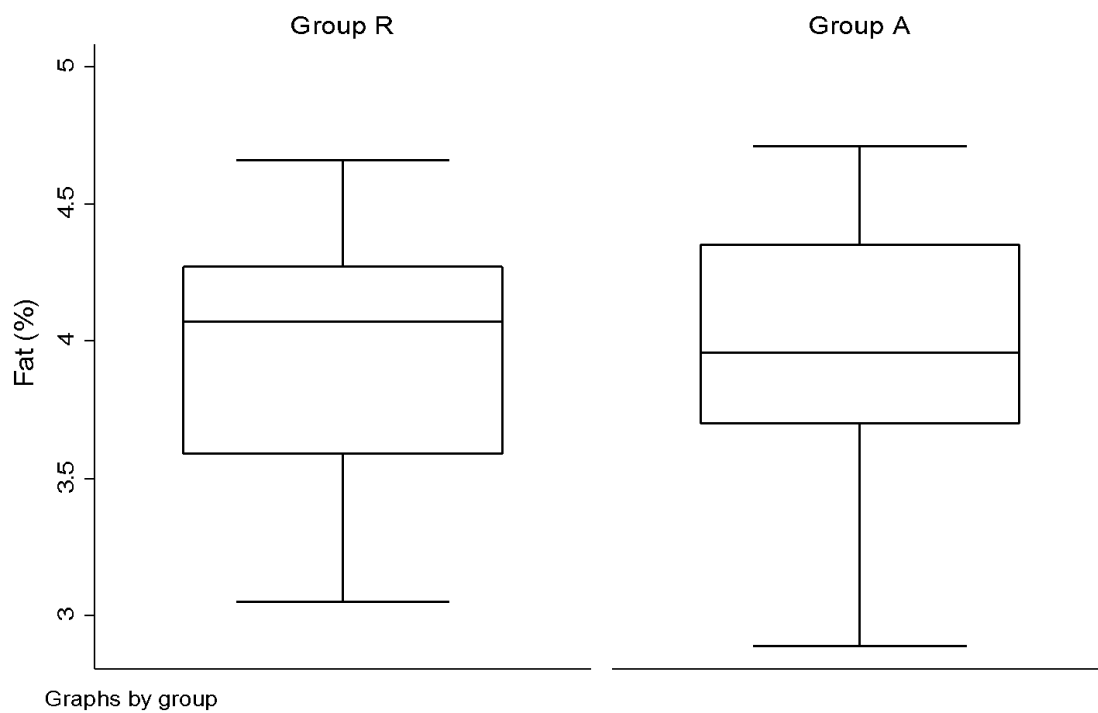


Figure 5.2 Box plot for milk fat percentage in first lactation for Group A (*ad libitum*) (n=39) and Group R (restricted) (n=38), (P=0.856).

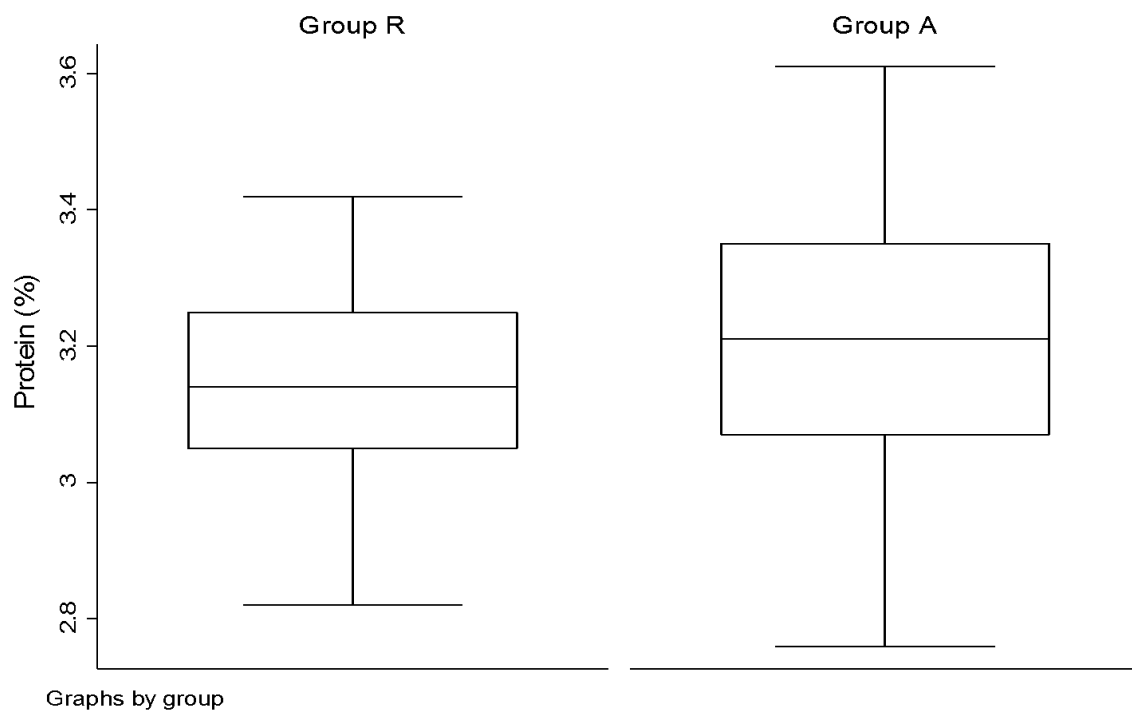


Figure 5.3 Box plot for milk protein percentage in first lactation for Group A (*ad libitum*) (n=39) and Group R (restricted) (n=38), (P=0.048).

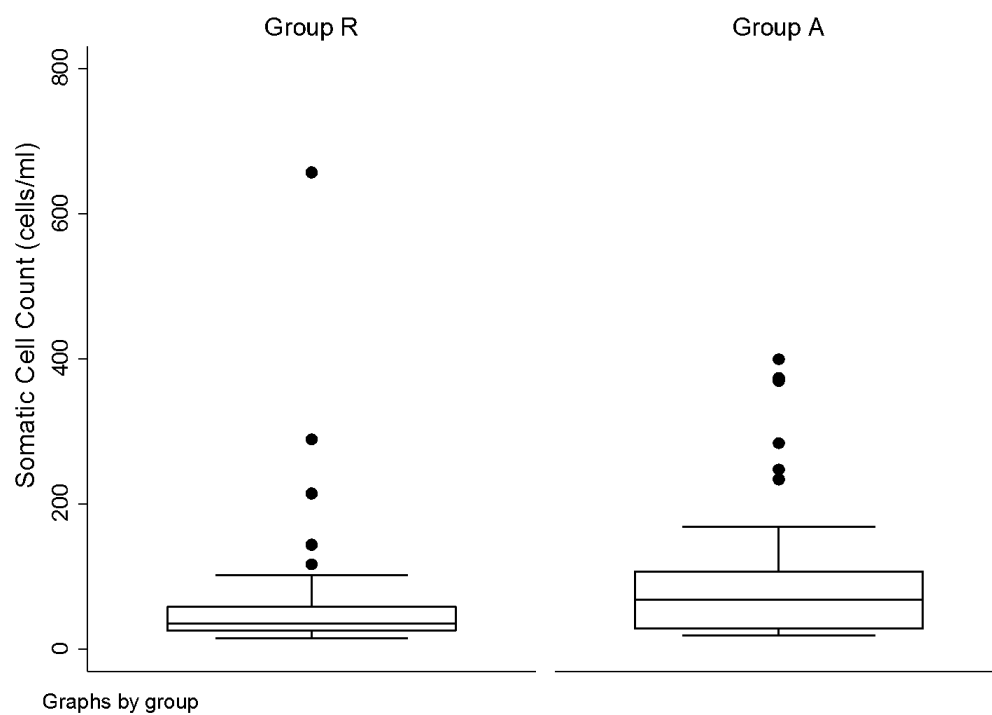


Figure 5.4 Box plot for somatic cell counts (cells/ml) in first lactation for Group A (*ad libitum*) (n=39) and Group R (restricted) (n=38), (P=0.035).

5.4 Discussion

5.4.1 Total Lactation, Actual 305-d Milk Yield and MilkBot Predicted 305-d Milk Yield

To the best of our knowledge, the present study is the first to evaluate and compare total lactation, actual 305-d milk yield and MilkBot predicted 305-d milk yield in heifers that received different milk replacer during pre-weaning period. However, this dataset was relatively small, thus decreasing the likelihood of detecting a significant association.

In the present study, the mean milk production per lactation was not different between Group A and Group R heifers. Similarly, the mean actual and MilkBot predicted 305-d milk yield also were not differed between the 2 groups. It may be speculated that the dataset was relatively small, thus decreasing the likelihood of detecting significant differences. However, the majority of published studies (Davis-Rincker et al., 2006; Aikman et al., 2007; Morrison et al., 2009; Raeth-Knight et al., 2009; Terre et al., 2009) have failed to show any significant effect of level of pre-weaning nutrition on first lactation milk yield. In contrast, other studies do find a significant positive relationship between level of pre-weaning nutrition and milk yield (Bar-Peled et al., 1997; Shamay et al., 2005; Drackley et al., 2007). Both Bar-Peled et al. (1997) and Shamay et al. (2005) compared whole milk with restricted milk replacer, while Drackley et al. (2007) documented significant increases in milk yield with increased feeding levels of milk replacer. Similarly, Moallem et al. (2010) stated that heifers fed *ad libitum* amounts of whole milk produced 10% more milk in their first lactation compared with those fed milk replacer during pre-weaning period. The improvements in milk yield observed in heifers provided whole milk during early life might be related to the greater total body growth or to physiological effects of whole milk on mammary gland development (Meyer et al., 2006).

5.4.2 Milk Fat Percentage and Milk Protein Percentage

To the best of our knowledge, the present study is the first to evaluate and compare milk fat and milk protein percentage in heifers that received different milk replacer feeding protocols during pre-weaning period.

In our study, milk fat percentage was not differed between Group A and Group R throughout the lactation period. However, milk protein percentage was higher in Group A than Group R over the lactation period.

Drackley et al. (2007), Raeth-Knight et al. (2009) and Davis Rincker et al. (2011) reported that there were no differences in milk fat and milk protein percentages found in their study. Average milk yield was significantly increased by 3.22 kg/day, whereas milk fat and protein percentages were significantly decreased by 0.2% and 0.1%, respectively (Shamay et al., 2005). However, Moallem et al. (2010) found that milk protein percentage was lower in the whole milk fed group than in the milk replacer fed group, whereas no effect of the 2% added protein was observed.

5.4.3 Somatic Cell Counts

To the best of our knowledge, the present study is the first to evaluate and compare somatic cell counts in heifers that received different milk replacer feeding regimes during pre-weaning period.

In this study, Group A heifers had nearly double the SCC compared to Group R heifers. High of SCC in these heifers might be caused by the contagious bacteria. The most common source of contagious bacteria is other infected heifers or cows; whereas environmental pathogens are most commonly isolated from recently calved and dry cows. However, in our study the

incidence of mastitis was very low and only 2 heifers were treated for mastitis (2.3%), (Chapter 4). In contrast, Drackley et al. (2007), Raeth-Knight et al. (2009) and Davis Rincker et al. (2011) reported no difference in SCC between groups in their studies on early calf nutrition.

In this study also, we found that SCC was positively related with AFC and milk fat percentage and negatively related to milk yield at first lactation. An increased AFC in heifers at any time point will affect their SCC. Heifers with high SCC at any time point will affect milk fat percentage. Meanwhile, heifers with high SCC at any time point will affect their milk yield; primarily due to the damage to milk producing tissue in the udder caused by mastitis pathogens and the toxins they produce, particularly when epithelial cells are lost. The positive correlation between SCC and milk fat percentage and negative correlation between milk yield and SCC were well documented by other researchers (Rupp & Boichard, 2000; Cinar et al., 2015).

Somatic cells in the milk of a healthy cow include 75 to 85% leucocytes and 15 to 25% epithelial cells (Barrett, 2002) and a $SCC \leq 100 \times 10^3$ cell/ml is considered healthy (Hillerton, 1999). SCC in milk is used as an indicator to monitor the degree of the udder health. An increase in SCC is widely considered as early emergence of inflammatory changes of mammary gland. In the Netherlands, a threshold of $>150,000$ cell/ml is commonly used to diagnose subclinical mastitis in heifers, whereas $>250,000$ cells/ml is used in multiparous cows (Sampimon et al., 2010).

5.4.4 *Variables Associated with Milk Yield (Dam)*

In the present study; body weight, heart girth and belly girth at calving were positively related to milk yield in first lactation. A previous study conducted by Moallem et al. (2010) showed similar correlations for body weight, heart girth and hip width at calving and milk production in the first lactation. Sieber et al. (1988) and Hoffman and Funk (1992) found a positive relationship between body weight at calving and first lactation milk production. Sejrsen et al. (2002) proposed that the positive correlation between body weight and milk production is mainly attributable to a positive relationship between growth rate capacity, which is related to the genetic potential and milk yield. Heifers that are selected for high milk yield have a higher growth capacity and calve with higher body weight. Macdonald et al. (2005) stated that body weight at calving and post-pubertal growth rate management were important in the first lactation milk production, but did not affect milk production in the subsequent lactations. In our study, the difference in body weight at calving was speculated because of nutritional management rather than by genetic potential and there was still a positive relationship between body weight at calving and milk yield.

In the present study, we found that age, body condition score and BHB values at calving were not associated with milk yield at first lactation. Storli et al. (2014) stated that young first lactation cows produce less milk than older first lactation cows but the effect of age levels off at around 33 months of age. This is consistent with results shown by others (Van Amburgh et al., 1998; Ettema and Santos, 2004).

5.4.5 *Variables Associated with Milk Yield (Calf)*

We demonstrated that milk yield of heifers that gave birth to bull calves was higher than those having heifer calves. Similarly, Graesboll et al. (2015) reported that dams produced more milk following the birth of a bull calf. They found a significantly higher milk production of 0.28% rather than our 12%. Cows that had bull calves in each of their first two parities produced significantly more milk than cows that produced heifer calf in the first parity and bull calf in the second parity. In contrast to our results and Graesboll et al. (2015), Hinde et al. (2014) reported that cows produced a female calf on their first parity produced significantly more milk on their second lactation, regardless of the sex of the calf on the second parity. We predicted to find similar results to Hinde et al. (2014), stated that Holstein experience a reduction in milk yield when having bull calves. However, this prediction was not supported by our data analysis, which showed those heifers' milk synthesis favour bull calves. According to Trivers hypothesis, dams may invest more in male offspring (measured in milk yield) because they profit relatively more from investing in bull calves than in heifer calves (Trivers and Willard, 1973). Trivers hypothesis also depicted that investment in female offspring will be more profitable than in male offspring when the individuals are in poor condition because the chance of producing competitive male offspring is low. Therefore, it can be speculated that the results from our study and Graesboll et al. (2015) suggest that British and Danish Holstein cattle are generally in good body condition because the heifers produce more milk for bull calves compared to US Holstein where they apparently invest more milk in heifer calves (Hinde et al., 2014). The reason for these differences in finding is not clear. However, further study should address this issue.

In our study, the birth weight of male calves was heavier than female calves. We found that birth weight of the calves did not affect milk yield. Afzal et al. (2007) also observed no influence of birth weight on milk yield for Nili Ravi buffaloes in Pakistan. Chew et al. (1981)

presented the relationship between calf birth weight and milk yield was linear and positive for birth weight between 23 and 50 kg. Birth weight more than 50 kg had a negative relationship with milk yield, because of high incidence of dystocia for heavy calves. Investigating calf sex second parity and birth weight was beyond the scope of our study. It may be speculated that the dataset was relatively small, thus decreasing the likelihood of detecting significant differences.

Findings may be a balance between a positive effect of male calf on yield and a negative effect of dystocia on yield and the balance depending on the relative impact of each in the dataset due to heifer body condition score, sire and sire selection.

5.4.6 MilkBot Parameters

To the best of our knowledge, the present study is the first to use MilkBot lactation model in heifers that received different milk replacer feeding protocols during pre-weaning period. Development of the MilkBot lactation model was based on the aim of identifying how management of dairy herds could influence the shape of lactation curves by producing parameters whose differences could be compared statistically (Ehrlich, 2013).

In the present study, the average estimated scale was 39.6 kg and there was no difference between Group A and Group R. Nemeckova et al. (2015) reported that scale lactation curve parameter was higher in the high milk yield group compared with the medium milk yield and the low milk yield groups. Scale values are a general measure of the productivity of breed, rises with parity in all breeds and varies between breeds with Holstein having the greatest scale values followed by Holstein crossbred and then other breeds.

In our study, Group R had greater estimated ramp (32 days) compared to Group A (29 days). Nemeckova et al. (2015) stated that ramp parameter was nearly the same between high milk yield, medium milk yield and low milk yield groups. Ramp is higher in heifers (indicating a slower rise in production after calving) (Ehrlich, 2013), lower for second parity and Holstein lactations rise more slowly (greater ramp) than other breeds.

Offset represents the difference between the recorded birth of the calf and the physiological start of lactation. Jerseys have higher offset than other breeds (Ehrlich, 2011) but no differences were seen between the groups in the present study.

In the present study, the average estimated decay was 0.001 per day and there was no significant difference between Group A and Group R animals. Decay should be lower in first parity animals (higher persistence in heifers) than mature cows.

The average estimated persistence was 917 days and did not differ between Group A and Group R animals in our study. Nemeckova et al. (2015) reported that persistence was longer in high milk yield compared to medium milk yield and low milk yield groups. Decay can be expressed as half-life, called persistence, corresponding approximately to the time in days for production to decrease by half in late lactation (Ehrlich, 2011).

In the present study, the average estimated peak milk was 35.0 kg and there was no difference between the 2 groups. Nemeckova et al. (2015) found that peak milk was higher in the high milk yield group compared to medium milk yield and low milk yield groups. Peak milk shows a similar pattern to scale and lower decay which is characteristic of heifers (Ehrlich, 2013). Buckley et al. (2003) reported that the higher the peak milk is, the higher the total milk yield.

The average estimated peak day was 83 days and did not differ between the groups in the present study. Nemeckova et al. (2015) stated that peak day occurred later after calving in the high milk yield group compared to medium milk yield and low milk yield groups. Peak day is not influenced by production level, relatively little variation among herds, fixed biologically and insensitive to management and environment. Muir et al. (2004) estimated that positive genetic correlations between 305-d milk yield and peak day was (0.63 ± 0.06) and persistency was (0.21 ± 0.06) , indicating that as yield increased, the interval from initiation of lactation to peak milk and persistency increased as well.

5.5 Conclusion

From our study, we can conclude that there was no effect of different pre-weaning feeding strategies that were applied during early life of heifer calves on their first lactation milk yield performance: total lactation, actual 305-d milk yield, MilkBot predicted 305-d milk yield, milk fat percentage, total protein yield, total fat yield and MilkBot parameters (scale, decay, persistence, peak milk and peak day) between Group A and Group R heifers.

However, several significant findings were observed in our study; for instance milk protein percentage and somatic cell counts were higher in Group A heifers and MilkBot parameter (ramp) was higher in Group R heifers.

Therefore, increasing the milk replacer level before weaning may decrease age at puberty, age at first service and age at conception but had no effect on milk yield performance.

Chapter 6

General Discussion

6.1 General Discussion

“Consumer expectations, economic pressures, an evolving regulatory framework, technological innovations and demographic shifts have contributed to the impetus for changes in the global dairy industry”, (Barkema et al., 2015). These changes have had and will likely continue to have, profound effects on the health and welfare of dairy cows and on management practices and systems for dairy herds. Over the last 30 years UK dairy herd size and lactational yield have increased, meanwhile herd numbers have decreased. The financial pressure exerted upon UK dairy farms, in term of farm gate milk prices, have forced dairy producers to either move away from dairying altogether or to reduce their cost of production. The cost of producing heifer replacements is a major contribution to overall farm overheads and optimising the stage in production may have long term benefits. The current study was designed to investigate and compare the impact of different pre-weaning feeding strategies that were applied during early life of heifers on their future potential performances in the first lactation milk yield.

The first objective of this thesis was to determine the effect of two different milk replacer feeding strategies in early life of heifer calves on the growth of heifers and their offspring by comparing their performance between *ad libitum* milk replacer fed calves (Group A) and restricted milk replacer fed calves (Group R) groups, (Chapter 3).

The feeding strategies resulted in no difference in body measurements at calving for example: wither height (WH), loin height, heart girth (HG), belly girth and crown rump length of the heifers between groups. There was about 12 weeks difference, not very large difference in term of overall energy or protein intake and compensatory growth may occur once the restricted group had access to more feed. The correlation between body weight (BW) and HG of heifers in this study was high ($r=0.79$), suggesting it is a valid proxy for BW.

In our study, BW at calving of heifers was 566 kg and 570 kg for Group A and Group R respectively. Despite a tendency for a higher initial growth rate in Group A, there was no difference in BW at calving between groups. Hence, there was no effect of different milk replacer feeding strategy during early life of the heifers on BW at calving. Meanwhile, there was a positive relationship between BW at calving and age at first calving (AFC) i.e. heifers that grew best and were heavier at any time point calved at a younger age. Our results support finding of Fisher et al. (1983). BW is closely related to age and BW at calving is in part determined by AFC, whereas AFC can be influenced by body size and the start of breeding (Moore et al., 1991).

Mean body condition score (BCS) of heifers at calving were 2.59 and 2.53 for Group A and Group R respectively and not different between groups. Thus, different pre-weaning feeding strategy in the early life of heifers did not influence their BCS at calving. Meanwhile, there was a positive relationship between BCS at calving and BW at calving and AFC.

In our study, the mean AFC was 25.0 months (751 days) and 25.4 months (761 days) for Group A and Group R respectively. Despite a tendency for an earlier conception in Group A, there was no significant difference in AFC between the 2 groups. This agrees with the findings of a number of other studies (Aikman et al., 2007; Drackley et al., 2007; Morrison et al., 2009; Terre et al., 2009). However, some studies have shown a 28 to 31 days reduction in AFC with heifer calves that were offered a higher feed level up to weaning (Bar-Peled et al., 1997; Raeth-Knight et al., 2009). Based on previous studies, the target for AFC for dairy heifers is 23-24 months (Hare et al., 2006). Gill and Allaire (1976) suggested that optimal AFC for total lifetime performance is 22.5 to 23.5 months.

Currently, the mean AFC in the UK is reported to be greater than 26 months (Brickell et al., 2009), representing a significant financial loss. Although, the *ad libitum* milk replacer fed

heifers reached all the reproductive Key Performance Indicators (KPI) (age at puberty, age at first service and age at conception) earlier than the restricted milk replacer fed heifers, the target AFC of 24 months was not met by either group. This suggests nutritional or reproductive management common to the two groups need to be improved. If the benefits of improved early life growth are to be realised, farm management practices must be optimised to ensure that heifers are served as early as possible after becoming eligible. Probably, the target should be all heifers are served within 21 days after achieving target BW or WH. This requires both regular monitoring of BW and WH of heifers and also active heat detection. In addition, BW and WH growth rate seem to be the fastest during the first 6 months of life and changing the raising rate during this period is the most efficient way to improve heifer growth performance (Kertz et al., 1998).

Our data showed no differences between groups in the body measurements of their calves on the day of calving (birth weight, heart girth, belly girth, wither height, loin height, crown rump length and hock fetlock length) of the proportion of calves of each sex.

Male calves were heavier than female calves. This has been observed in several studies (Kertz et al., 1997; Dhakal et al., 2013). The effect of the dam's nutritional supply on the calf has long been a topic of discussed (Holland and Odde, 1992). Many researchers report no influence of early and late pregnancy nutrition on the offspring's birth weight. Eckles (1916) stated that body weight of a calf at birth was not influenced by the ration received by the dam during gestation unless severe nutritional deficiencies existed. The birth weights of calves born to heifers still growing are reduced in comparison with non-growing heifers and, heavier, older cows (Holland and Odde, 1992). Thus, calves born to very young heifers (20.3 – 22.0 months) had a comparatively lower birth weight compared to calves born to young heifers (22.0 – 23.5 months). It is suggesting that the intrauterine environment may limit foetal growth due to competition for nutrients with the dam (Wathes et al., 2008).

The second objective of this thesis was to determine the effect of the two different milk replacer feeding strategies in early life of heifer calves on their future fertility and health performance, (Chapter 4).

No difference was observed between the 2 groups of heifers on reproductive parameters (calving to first oestrous, calving to first service, calving to conception, calving interval and number of service). Gilmore et al. (2011) reported that conception rate to first service, incidence of atypical progesterone profiles and 100 days in-calf rate were not affected by nutritional composition of the diets in cows.

An early onset of oestrous cyclicity in the heifers increases the probability of an early insemination after calving, shortens the interval from calving to conception, increases conception rate and reduces the number of services per conception (Darwash et al., 1997). Relative to multiparous cows, first calved heifers have on average a greater incidence of delayed interval from calving to first ovulation postpartum (Tanaka et al., 2008). Reproductive performance is influenced by many factors, including nutritional and management practices but the decline in dairy cow fertility has also been associated with an increased genetic capacity for milk production achieved by the replacement of the British

Friesian by the North American Holstein (Royal et al., 2000). The percentage of Holstein genes in the UK dairy herd has increased from approximately 0% in 1975 to 80% in 1998 (Royal et al., 2000). Same thing has also happened in the Netherlands, (Hoekstra et al., 1994). However, Buckley et al. (2003) reported that the proportion of Holstein genes had no effect on pregnancy rate to first service and higher milk yield was associated with improved conception rates to first service. Feeding heifers more early in development does not appear to be a strategy that could aid in reducing this fertility decline.

Other factors, for example management changes such as increases in herd size or the increases in negative energy balance in early lactation, associated with higher milk yields and inadequate nutrient intake. A decline has occurred in the observed expression, intensity and detection of animals in oestrus (Van Eerdenburg et al., 1996; Kerbrat and Disenhaus, 2004) and poor heat detection is a major contributor to decreased reproductive performance in modern high yielding dairy cows (Reimers et al., 1985) due to shorter and less intense oestrus expression (Lopez et al., 2004). Lyimo et al. (2000) reported that maximum oestradiol concentrations which are influenced by negative energy balance were related to total oestrus expression.

Commencement of luteal activity (CLA), pregnane profiles, luteal phase length and inter-luteal interval in the first reproductive cycle after calving; did not vary between treatment groups. Hence, endocrine fertility parameters of the heifers were not influenced by the different milk replacer feeding strategy between the 2 groups during their early life. The time to CLA recorded in the present study was in general agreement with other studies that previously reported by Darwash et al. (1997), Royal et al. (2000), McCoy et al. (2006), Law et al. (2009) and Gilmore et al. (2011). CLA occurs 4 to 5 days after first ovulation (Darwash et al., 1997) and is a direct measurement of the resumption of ovarian activity after calving. Whilst there was a trend for more Group R heifers to exhibit delayed ovulation (DOV1 profile). However, other profiles (normal, DOV2, PCL1 and PCL2) were not different among the groups. There was a higher incidence of all abnormal pregnane profile types, particularly delayed ovulation (DOV1 and DOV2) in the current study. This may be related to nutrition condition during early postpartum. But the low occurrence of PCL (PCL1 and PCL2) could be explained by the low number of postpartum problems that happen in the heifers. The luteal phase was shorter in the first cycle postpartum than in the second cycles but was unaffected by early life feeding strategy. The length of second inter-luteal interval was shorter compared

to the length of first inter-luteal interval and there was again no difference between the groups in our study. Opsomer et al., (2000) stated cows with clinical endometritis had delayed resumption of ovarian activity and more prolonged luteal phases. There was very low incidence of endometritis in our study so this relationship could not be investigated. Meanwhile, Royal et al. (2002) reported that cows with a genetically longer interval to the start of the first cycle (CLA) had a longer calving to first service interval and a longer calving interval. A later start of ovulation and longer calving to first service and calving interval will decrease the total milk production per cow and also herd profitability.

According to Jorritsma et al. (2003) negative energy balance (NEB) has been determined as an underlying causal factor of poor reproductive performance in high yielding dairy cows and also has been associated with a delay in the CLA (Jolly et al., 1995), an extended interval to first service (Butler et al., 1981) and decreased conception rates (Domecq et al., 1997). NEB affects ovarian function through a decrease in the maximum diameter of ovarian dominant follicles (Lucy et al., 1991; Mackey et al., 1999). Thus, smaller dominant follicles produce less oestradiol, suppressing the pulsatile secretion of LH (Butler, 2001) and decreasing ovarian responsiveness to LH (Butler, 2001). Later, these events increase the proportion of follicles that fail to ovulate (Mackey et al., 1999). The expression of oestrus is positively correlated and controlled by plasma oestradiol concentration (Lyimo et al., 2000). Thus, larger pre-ovulatory follicles have been associated with higher oestradiol concentrations which potentially increase the intensity of oestrus expression (Lyimo et al., 2000). However, Lopez et al. (2004) stated that high yielding dairy cows had lower oestradiol concentrations compared to lower yielding dairy cows despite having larger pre-ovulatory follicles.

During our study, we observed that the majority of our heifers' BCS was between 2.75-3.00 during pre-partum and was between 2.50-2.75 during postpartum period. Cows should calve down in a BCS of 2.75-3.00 and not lose more than 0.50 of a unit of BCS between calving

and first service (Overton and Waldron, 2004). Our results support this recommendation. Cows that calve down in poor BCS (<2.50) are more likely to have a prolonged anoestrous period; too low LH pulse frequency and reduced concentrations of oestradiol which are ineffective to induce an LH surge and ovulation. In addition to that, cows in poor BCS after calving have decreased diameter of the dominant follicle. Therefore, monitoring of BCS score from calving to first service is an important aspect of reproductive management. BCS changes are good indicators of energy balance and reflect milk yield and dry matter intake.

We found no relationships between AFC and subsequent reproductive parameters and pregnane profiles. Similarly, Simerl et al. (1992) reported that no effect of calving age on any measure of reproductive performance during the first lactation. Heifers calving for the first time at 25 to 26 months tend to have lower subsequent calving intervals compared to younger and older AFC groups (Evans et al., 2006). Thus, both early (<700 days) and late (>751 days) calving heifers had lower conception rates in the first lactation in comparison with those calving between 700 and 750 days (Ettema and Santos, 2004). Study by Eastham (2012) using data on a large proportion of the UK dairy herd calving for the first time and with AFC in the range between 21 to 42 months. They concluded that those calving at 23 to 25 months had the lowest calving intervals after each lactation compared to animals that calving at >36 months.

In our study, there were no animals required assistance during the parturition and few health issues were reported. Therefore, different pre-weaning feeding strategy that applied during early calves' life did not have effect on health performance of the animals in both groups.

In the present study, we also found that there was no difference in β -hydroxybutyrate (BHB) values from calving to 30 weeks postpartum between Group A and Group R heifers. The peak prevalence of subclinical ketosis (SCK) occurred at 14 weeks of postpartum (between

99-100 days of postpartum) and Group R heifers had high incidence of SCK compared to their counterpart although no heifer had clinical ketosis (CK) during the study period. Generally, more than 90% of SCK cases in large population surveys across multiple farms occur during the first and second months of postpartum, with the former containing the peak prevalence (Duffield et al., 1997; Suthar et al., 2013). Occurrence of SCK at 14 weeks of postpartum in our study was noticed at different time of the year for different groups, so it was not due to one difference in nutrition. However, different pre-weaning feeding strategy during early life of the heifers may not directly be the main effect. It can be postulated that heifers developing SCK within one week postpartum have experienced extremely poor adaptation to NEB through calving and into lactation, whereas heifers developing SCK after the first week postpartum may have better adapted to the effects of decreased DMI during and immediately postcalving but are not able to sustain energy stores for the increase in milk production in early lactation. Therefore, we hypothesise that other factors such as individual variation, genetic, BCS or interaction among them could be the causal factor. Individual variations are the differences that may vary from one individual to another in the same species of living organisms; for example different animals have different level of acceptance to stress or metabolic disorders. Every living thing contains the genetic material that makes up DNA molecules. This material is passed on when organisms/animals reproduce. Therefore, some good and bad genetic material can be transferred that influence the system of the body. BCS prior to calving is also an important risk factor for subsequent development of SCK during lactation. Duffield et al. (1998b) reported that fat cows ($BCS \geq 4.00$) had higher BHB concentrations postcalving and were also at higher risk of developing SCK compared to cows in moderate and thin body condition prior to calving (Duffield et al., 1998a).

Animals that affected by these metabolic disorders are more prone to anoestrous and reduced conception rate to artificial insemination (Grohn and Rajala-Schultz, 2000; Lucy 2001;

Lopez-Gatius et al., 2002). BHB and NEFA also may negatively affect oocytes and corpus luteum because these metabolites are elevated in the follicular fluid of dominant follicles (Leroy et al., 2004). Both BHB and NEFA may only be detrimental in the presence of low follicular concentrations of glucose (Leroy et al., 2006). However, in our study we did not measure glucose either in plasma or follicular fluid.

The third objective of this thesis was to determine the effect of different milk replacer feeding strategies in early life of heifer calves on their first lactation performance by comparing milk parameters between the 2 groups using the MilkBot lactation model, (Chapter 5).

There were no differences between Group A and Group R in total lactation, actual 305-d milk yield, MilkBot predicted 305-d milk yield, milk fat percentage, total protein and total fat yield. However, heifers in Group A had higher milk protein percentage and somatic cell counts (SCC). MilkBot parameters describe the lactation curve and, there were no differences in estimated scale, decay, persistence, peak milk and peak day between Group A and Group R. However, estimated ramp was higher in Group R suggesting the increase in yield after calving was slower in this group. Heifers have a slower rise in milk production after calving than cows (Ehrlich, 2013), but this is the first report of differences between groups based on early life feeding. Previous studies have shown that second parity and Holstein lactations rise more slowly (greater ramp) than other breeds. The majority of published studies (Davis-Rincker et al., 2006; Aikman et al., 2007; Morrison et al., 2009; Raeth-Knight et al., 2009; Terre et al., 2009) and also our study have failed to show any significant effect of level of pre-weaning nutrition on first lactation total milk yield. However, several studies find a significant positive relationship between level of pre-weaning nutrition and milk yield (Bar-Peled et al., 1997; Shamay et al., 2005; Drackley et al., 2007). Both Bar-Peled et al. (1997) and Shamay et al. (2005) compared whole milk with restricted milk replacer, while Drackley

et al. (2007) documented significant increases in milk yield with increased feeding levels of milk replacer.

We found a positive relationship between BW at calving and milk yield at first lactation. Heifers that grew best and were heavier at any time point performed better in milk yield. Several studies showed a positive relationship between BW at calving and first lactation milk yield (Clark & Touchberry, 1962; Fisher et al., 1983; Keown & Everett, 1986). According to Macdonald et al. (2005), BW at calving and post-pubertal growth rate is important for first lactation milk production but had no effect on subsequent milk production. In our study, 4 heifers (2 heifers from each group) that had BW at calving >660 kg showed no effect on their lactation performance (>9,000 kg). This is in agreement with Grummer et al. (1995) who reported that a BW at calving >660 kg did not enhance lactation performance.

On the other hand, there was no relationship between BCS at calving and first lactation milk yield in our study. Carson et al. (2002) found that lighter animals with a lower BCS at calving (2.80 compared with about 3.50 in other groups) produced slightly less milk in the first lactation but had better fertility. They calved for a second time sooner and produced approximately a similar milk output over 2 lactations.

From our study, we found a relationship between AFC and somatic cell counts (SCC) but not milk yield or milk component concentration. Heifers that calved later at any time point, their SCC will be affected. Heinrichs and Vazques-Anon (1993) reported that first lactation milk yield was reduced when heifers calved before 2 years of age. Other studies have shown that AFC can affect first lactation milk yield with both early and late calving ages having less production (Ettema and Santos, 2004). Milk components were also reduced when AFC was decreased; especially fat concentration in the milk (Pirlo et al., 1997; Ettema and Santos, 2004) but protein percentage was higher (Pirlo et al., 1997). However, modelling has

suggested that a reduction of AFC from 24 to 22 months has a negative effect on the first lactation milk yield and milk fat production (Pirlo et al., 2000). Longer AFC is more costly due to the longer time heifers are kept before entering the milking herd and a smaller number of animals are available for sale for the same replacement rate. Pirlo et al. (1997) stated that, reluctance to reduce AFC is attributable to the belief that early calving is detrimental to milk yield and longevity. They concluded that decreasing AFC to 23-24 months was the most profitable procedure, but not less than 22 months (except in cases of low milk prices and high rearing costs). Increasing rearing costs by feeding more milk replacer in the current study did not give any benefit on reducing AFC so strategies that reduce AFC by increasing growth rate 12 weeks of age or later fertility are needed to meet this objective.

We showed that milk yield of heifers that gave birth to bull calves was higher than those having heifer calves. This agrees with previous study by Graesboll et al. (2015). However, Hinde et al. (2014) found that Holstein experience a reduction in milk yield when having bull calves. The reason for these differences in finding is not clear. This thesis forms the basis of a database of lifetime performance data of a cohort of 88 pedigree Holstein dairy heifers with relatively low genetic variation. Therefore, further study to determine the performance of these animals throughout their productive lives is crucial.

6.2 Limitations of the Study

The present study enrolled animals over 2 years due to using a single 200 cow farm. It involved physical measurements, sampling, data collection and also laboratory works for each individual animal in *ad libitum* and restricted groups from 8 weeks prior to predicted calving until 30 weeks of postpartum. Long term studies of this type are difficult to conduct,

quite expensive, time consuming and suffer from data loss due to involuntary culling and also death of the animals during the study.

Our dataset was relatively small especially for fertility and milk yield data, therefore decreasing the likelihood of detecting significant differences. Many studies do not have enough power to appropriately test a hypothesis related to milk yield as an outcome of effects of nutrition, growth and development (Soberon and Van Amburgh, 2013).

6.3 Recommendations for Further Research

Further research is needed to determine and confirm differences in performance between groups for long term effect of early nutrition throughout their productive lives. The next phase (second/third parity) would be to understand the long term effect on growth, mature body weight, fertility, milk yield and health of the animals between the 2 dietary groups. Thus, there are many factors that may involve for instance, feeding system and level, system of milk production, cow's genetic background and also parity. Determining if there are any differences in subsequent performance related to heifer growth parameters measured in this study, regardless of the initial postnatal nutritional treatment would be worthy of study.

6.4 Overall Conclusion

Finally, from our study we can conclude that there was no large effect of different pre-weaning feeding strategies during early life of heifers on their growth, offspring, fertility, health and milk yield between *ad libitum* (Group A) and restricted (Group R) animals during their first lactation period.

On the other hand, several significant findings were observed during our study; heifers in Group A had higher milk protein percentage and higher SCC. Meanwhile, heifers in Group R had higher number of DOV1 profile, higher incidence of SCK and higher in estimated ramp. Another finding was heifers produced more milk following birth of a bull calf regardless of groups. However, overall the size of these differences was small and would not yet justify changing early nutrition to *ad libitum* feeding on a performance and financial basis with the analysis included in this thesis.

Chapter 7

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Appendix A

Tables

Table A.1 Body condition scoring chart for Holstein cows from Edmonson et al., 1989.

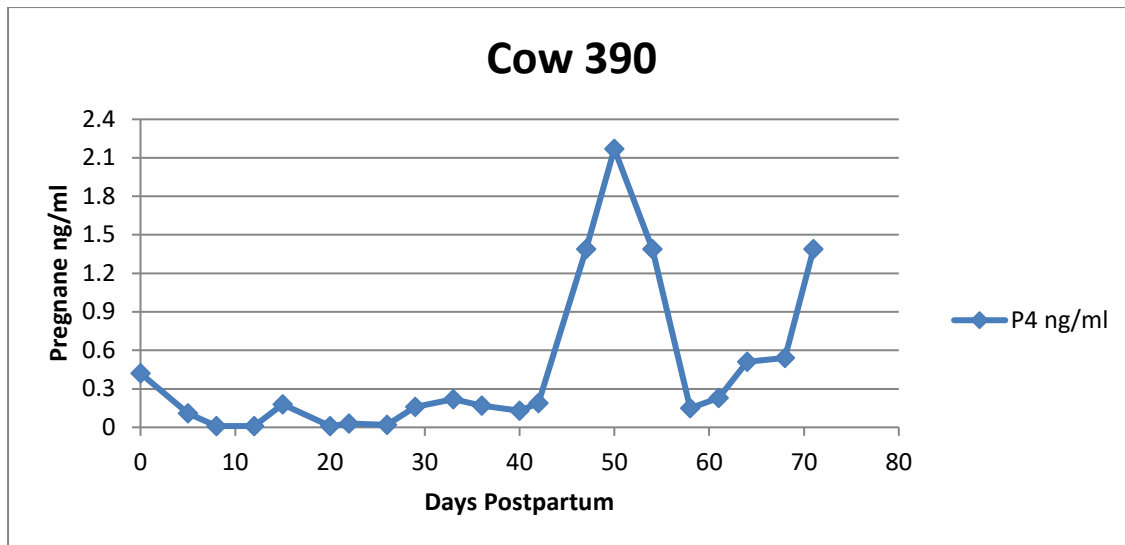
	SCORE	Spinous processes (SP) (anatomy varies)	Spinous to Transverse processes	Transverse processes	Overhanging shelf (care - rumen fill)	Tuber coxae (hooks) & Tuber ischia (pins)	Between pins and hooks	Between the hooks	Tailhead to pins (anatomy varies)
SEVERE UNDERCONDITIONING (emaciated)	1.00	individual processes distinct, giving a saw-tooth appearance	deep depression	very prominent; > 1/2 length visible	definite shelf, gaunt, tucked	extremely sharp, no tissue cover	severe depression, devoid of flesh	severely depressed	bones very prominent with deep "Y" shaped cavity under tail
	1.25								
	1.50								
FRAME OBVIOUS	1.75			1/2 length of process visible					
	2.00	individual processes evident	obvious depression	between 1/2 to 1/3 of processes visible	prominent shelf	prominent	very sunken		bones prominent "U" shaped cavity formed under tail
	2.25								first evidence of fat
FRAME & COVERING WELL BALANCED	2.50	sharp, prominent ridge		1/3 - 1/4 visible	moderate shelf		thin flesh covering	definite depression	
	2.75			< 1/4 visible	slight shelf	smooth	depression	moderate depression	bones smooth, cavity under tail shallow & fatty tissue lined
	3.00		smooth concave curve	appears smooth, TP's just discernable		covered	slight depression	slight depression	
FRAME NOT AS VISIBLE AS COVERING	3.25	smooth ridge, the SP's not evident	smooth slope	distinct ridge, no individual processes discernable					
	3.50			smooth, rounded edge	none	rounded with fat	sloping	flat	bones rounded with fat and slight fat-filled depression under tail
	3.75	flat, no processes discernable	nearly flat	edge barely discernable		buried in fat	flat		bones buried in fat, cavity filled with fat forming tissue folds
SEVERE OVERCONDITIONING	4.00								
	4.25								
	4.50								
	4.75	buried in fat	rounded (convex)	buried in fat	bulging		rounded	rounded	
	5.00								

Table A.2 Rumen fill score and appearance of paralumbar fossa from Zaaijer and Noordhuizen, 2003.

Rumen fill score	Appearance of paralumbar fossa		
	Relationship to the transverse process	Relationship to the last rib	Shape
1	Cavitates a hand's width inside under the transverse processes	Cavitates a hand's width behind it	Empty rectangle
2	Cavitates less than a hand's width inside under the transverse processes	Cavitates a hand's width behind it	Triangle
3	Falls about a hand's width vertically down and then bulges out	Cavitates less than a hand's width behind it	
4	Arches out immediately below it	Skin is covering the area behind it	Bulges out directly
5	Transverse processes not visible	Last rib not visible	Rumen almost obliterates it

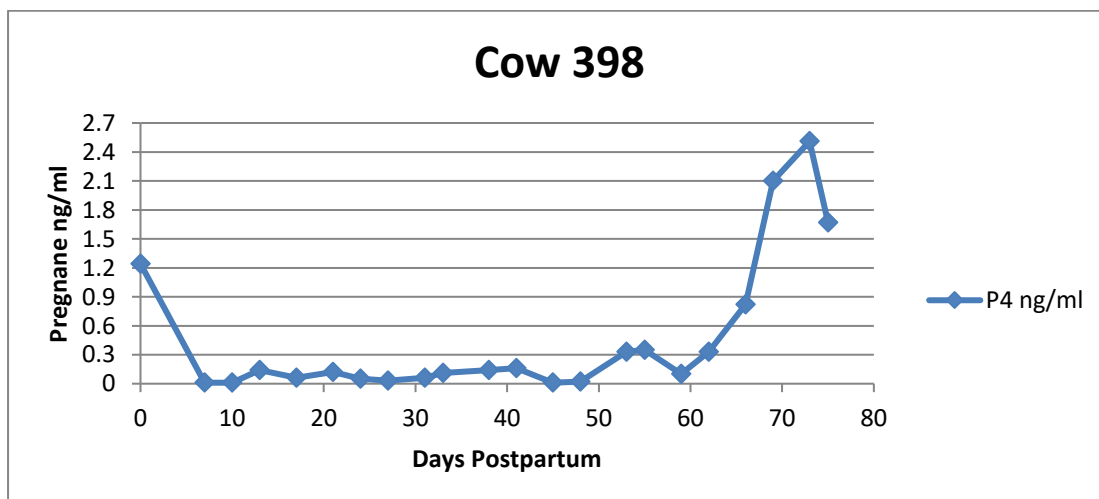
Appendix B

Pregnane Profiles



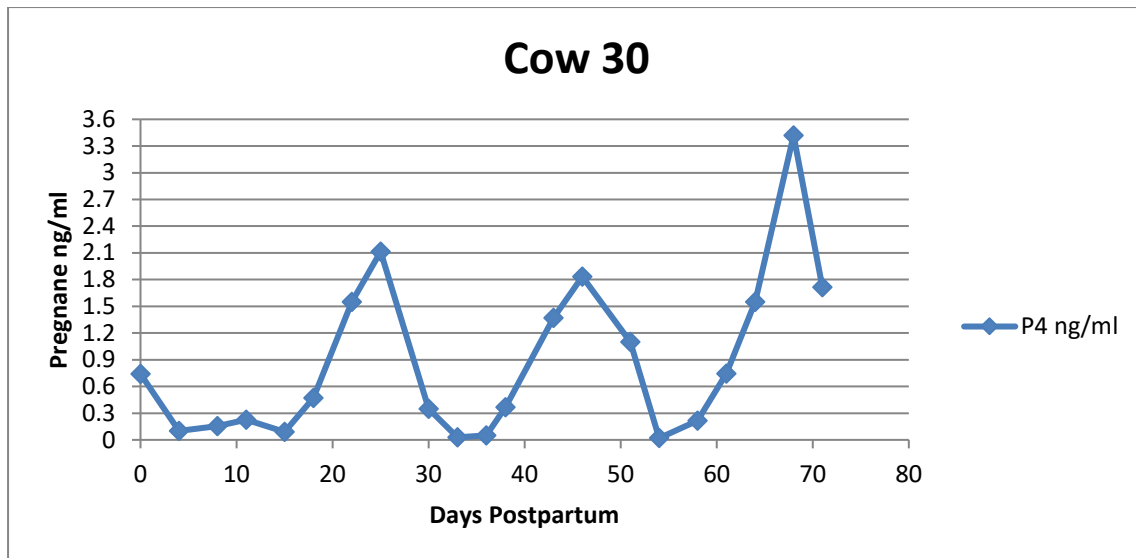
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 47; 1.39ng/ml).
- Luteal phase : $(T2 - T1 + 3.50)$; $(54 - 47) + 3.5 = 10.5$ days
- Inter-luteal interval : $(T6 - T5 + 3.5)$; $(61 - 58) + 3.5 = 6.5$ days
- Commencement of luteal activity : $(T1 - 1.75)$; $(47 - 1.75) = 45.25$ days



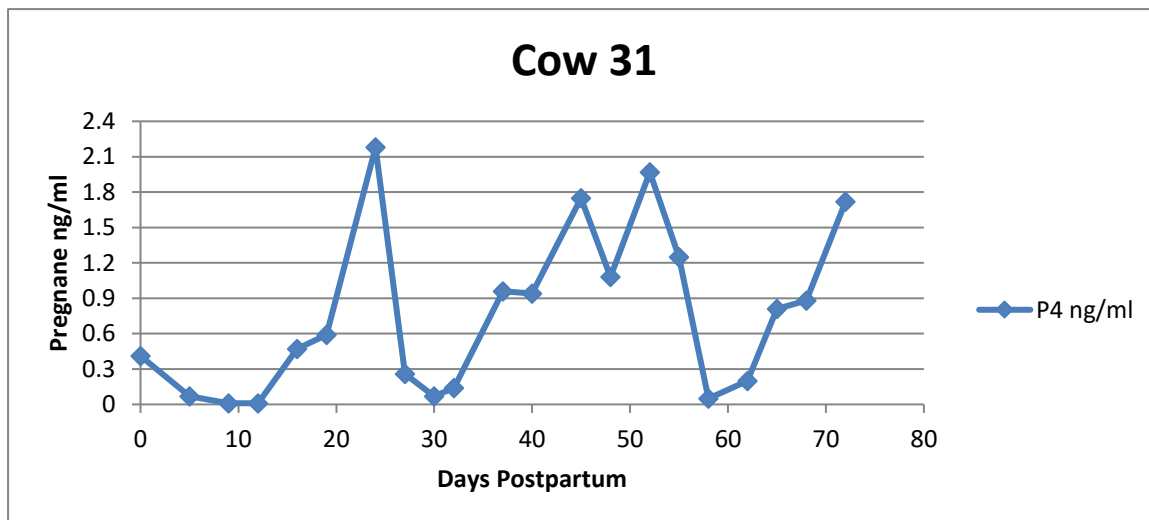
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 53 postpartum; 0.33ng/ml.
- Luteal phase : $(T2 - T1 + 3.5)$; $(55 - 53) + 3.5 = 5.5$ days
- Inter-luteal interval : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(53 - 1.75) = 51.25$ days



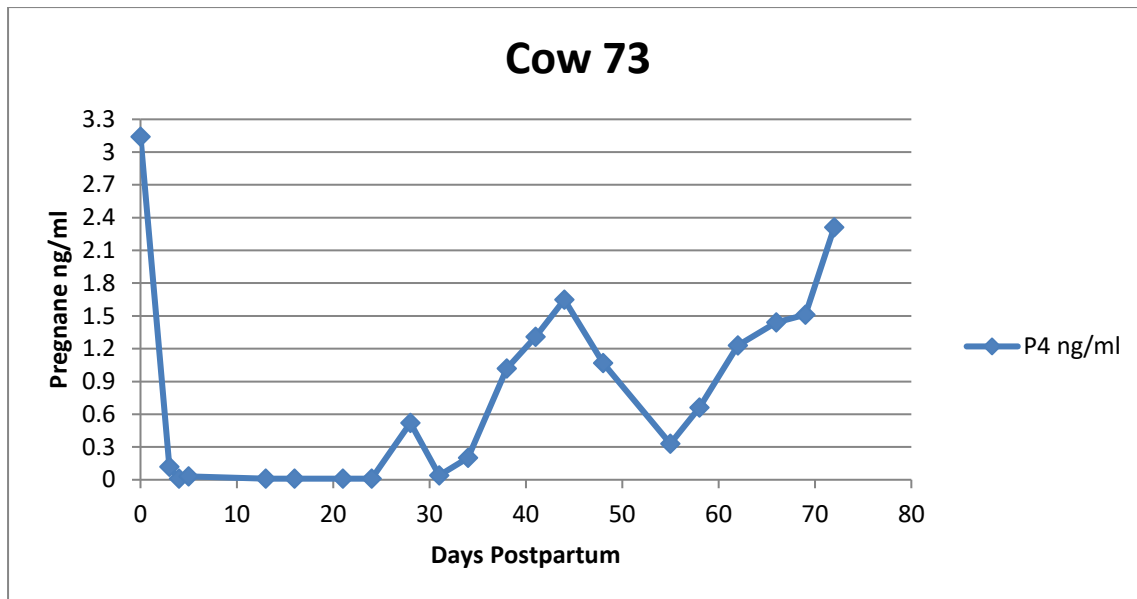
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.47ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(30 - 18) + 3.5 = 15.5$ days
- Inter-luteal interval : $(T6 - T5 + 3.5)$; $(36 - 33) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(51 - 38) + 3.5 = 16.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(58 - 54) + 3.5 = 7.5$ days



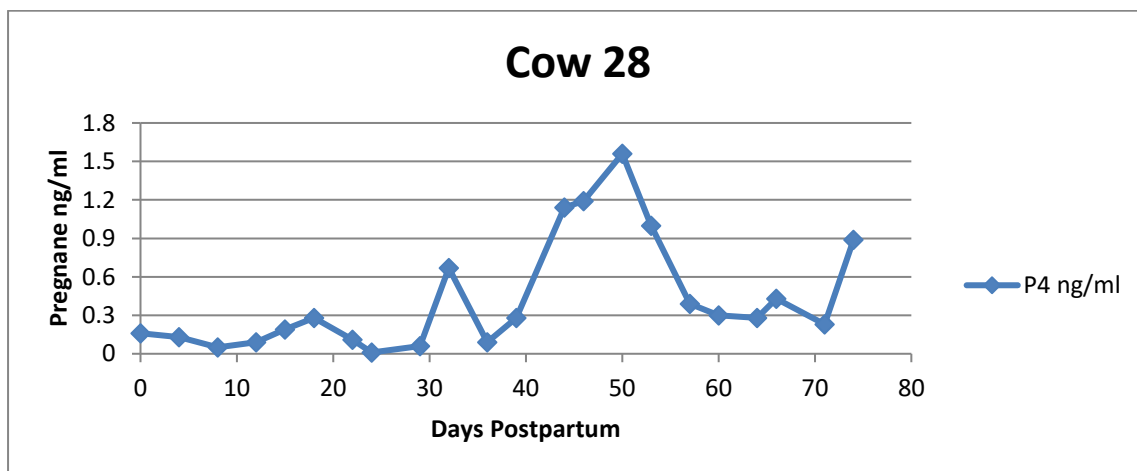
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 16; 0.47ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(24 - 16) + 3.5 = 11.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(32 - 27) + 3.5 = 8.5$ days
- CLA : $(T1 - 1.75)$; $(16 - 1.75) = 14.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(55 - 37) + 3.5 = 21.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(62 - 58) + 3.5 = 7.5$ days



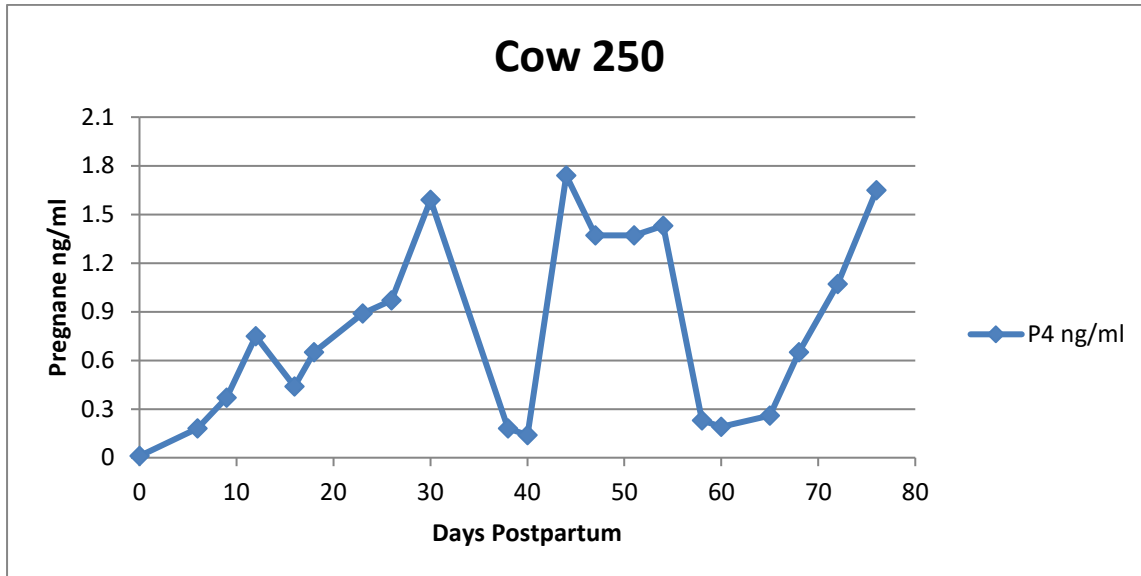
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 28; 0.52ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(34 - 31) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(28 - 1.75) = 26.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(72 - 38) + 3.5 = 37.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$;



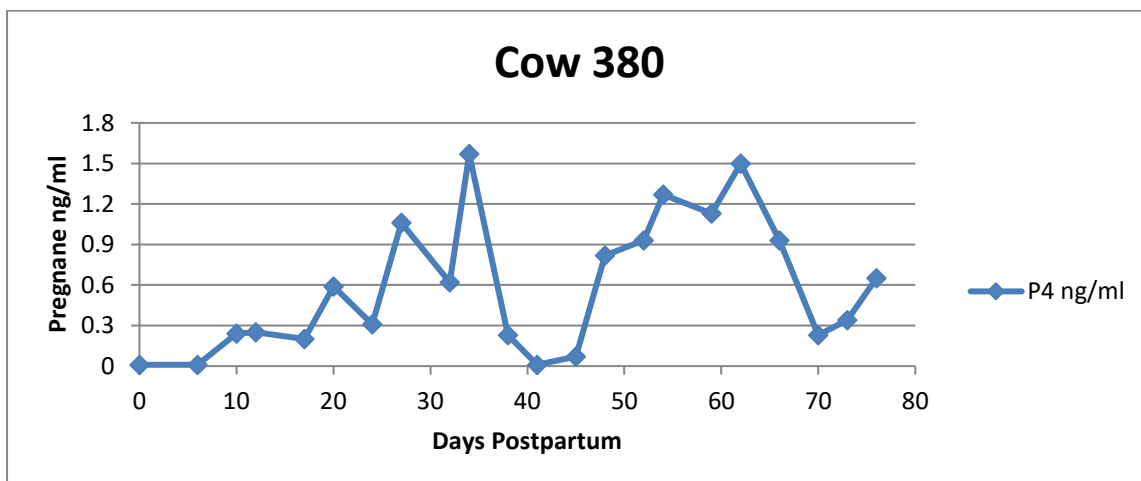
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 32; 0.67ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(39 - 36) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(32 - 1.75) = 30.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(60 - 44) + 3.5 = 19.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



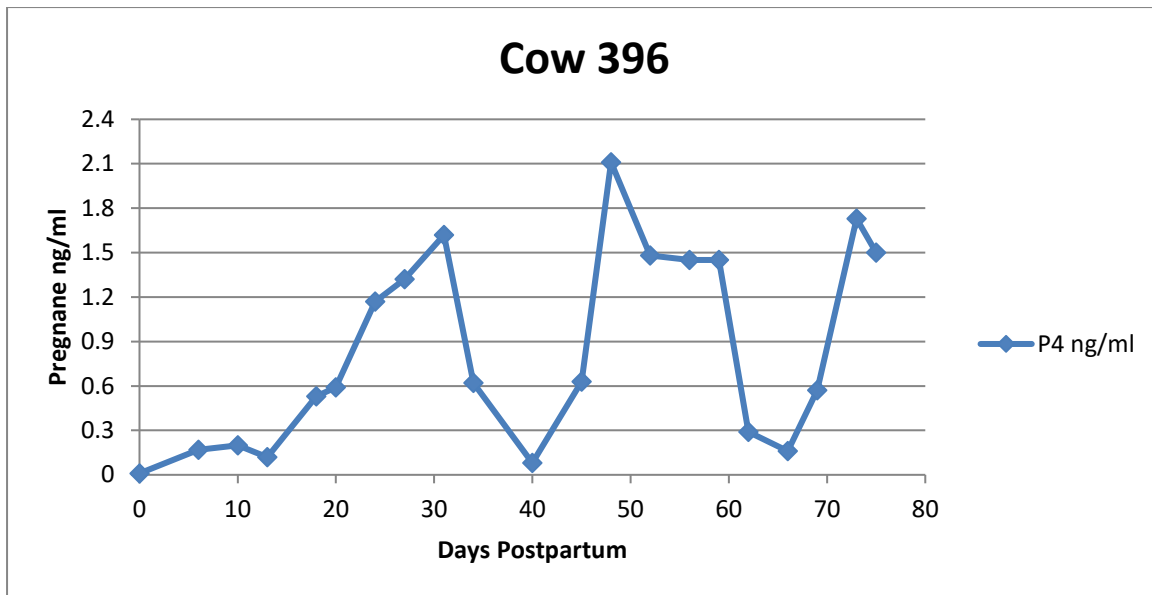
PCL1

- First rise of pregnane (>0.3ng/ml) occurred at day 9 postpartum; 0.37ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(30 - 9) + 3.5 = 24.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(40 - 38) + 3.5 = 5.5$ days
- CLA : $(T1 - 1.75)$; $(9 - 1.75) = 7.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(54 - 44) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(65 - 58) + 3.5 = 10.5$ days



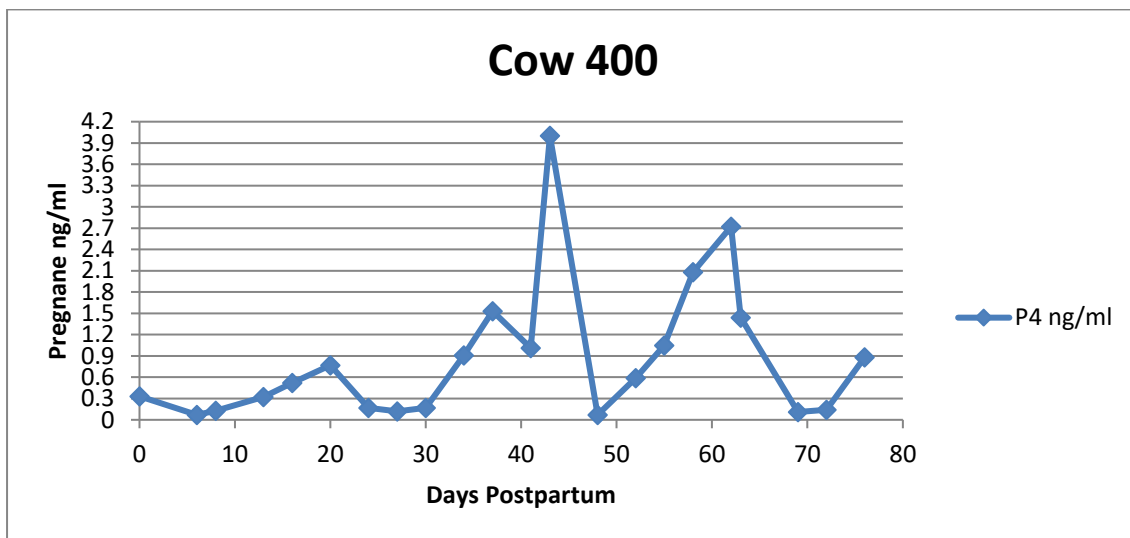
PCL2

- First rise of pregnane (>0.3ng/ml) occurred at day 20 postpartum; 0.59ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(34 - 20) + 3.5 = 17.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(45 - 38) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(20 - 1.75) = 18.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(66 - 48) + 3.5 = 21.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



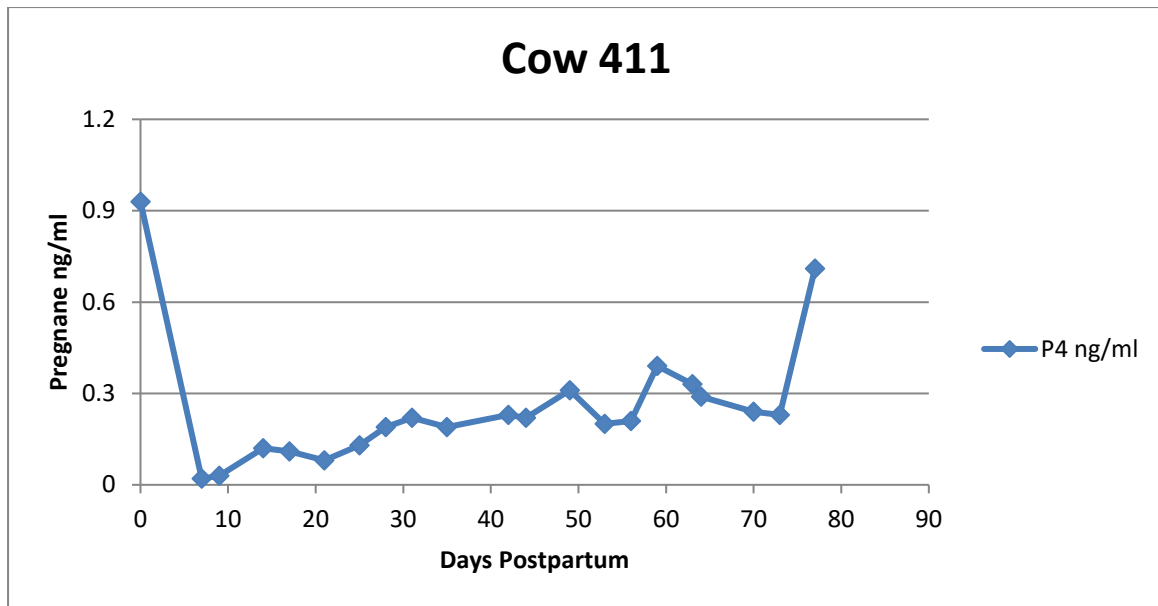
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.53ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(34 - 18) + 3.5 = 19.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(59 - 45) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(66 - 62) + 3.5 = 7.5$ days



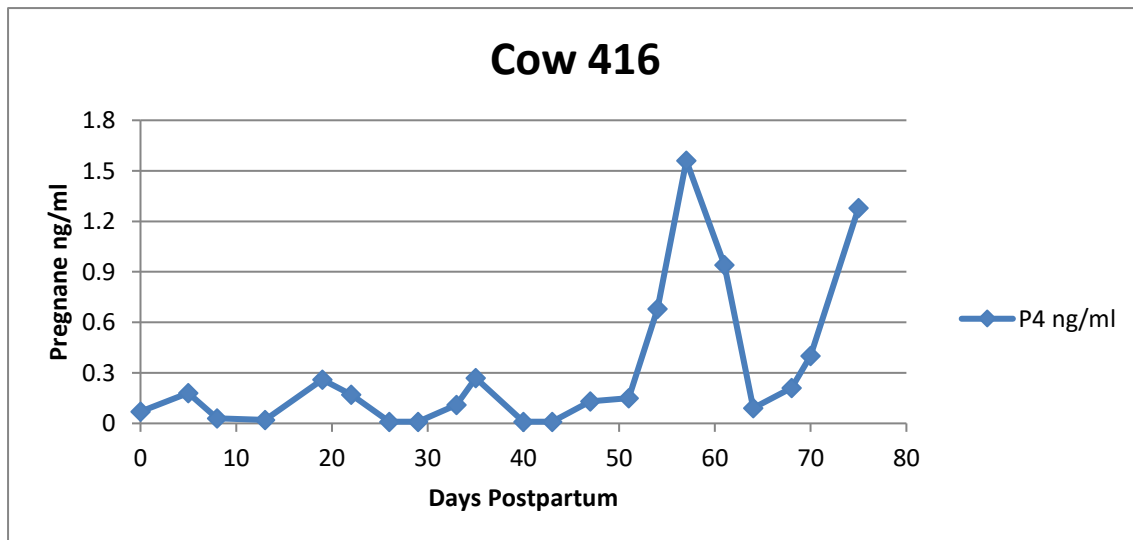
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 13; 0.32ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(20 - 13) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(30 - 24) + 3.5 = 9.5$ days
- CLA : $(T1 - 1.75)$; $(13 - 1.75) = 11.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(43 - 34) + 3.5 = 12.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



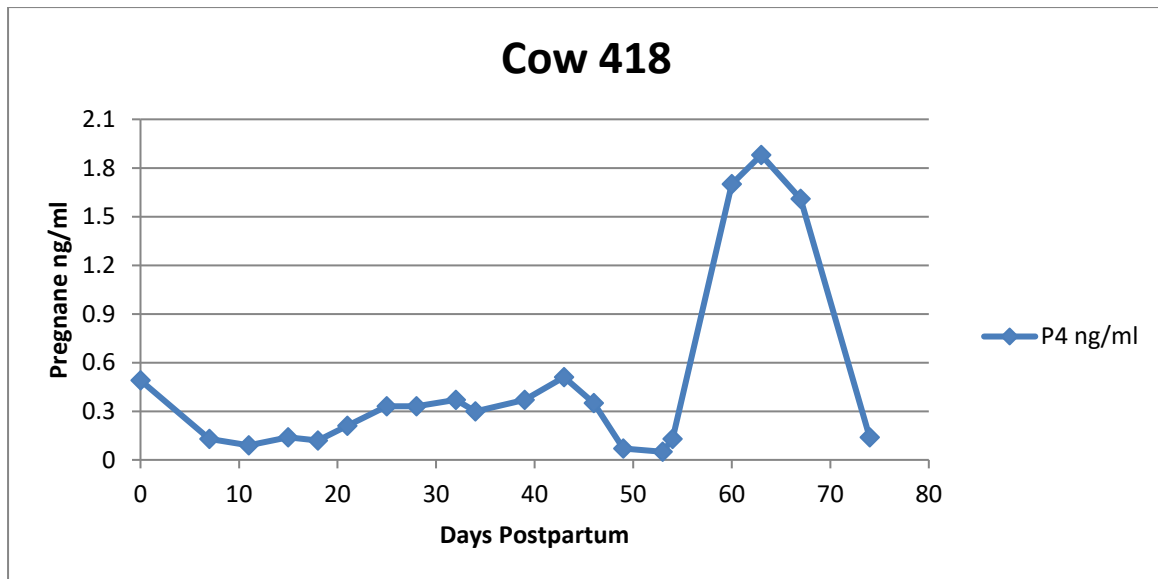
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 49 postpartum; 0.31ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(56 - 53) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(49 - 1.75) = 47.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(63 - 59) + 3.5 = 7.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(73 - 64) + 3.5 = 12.5$ days



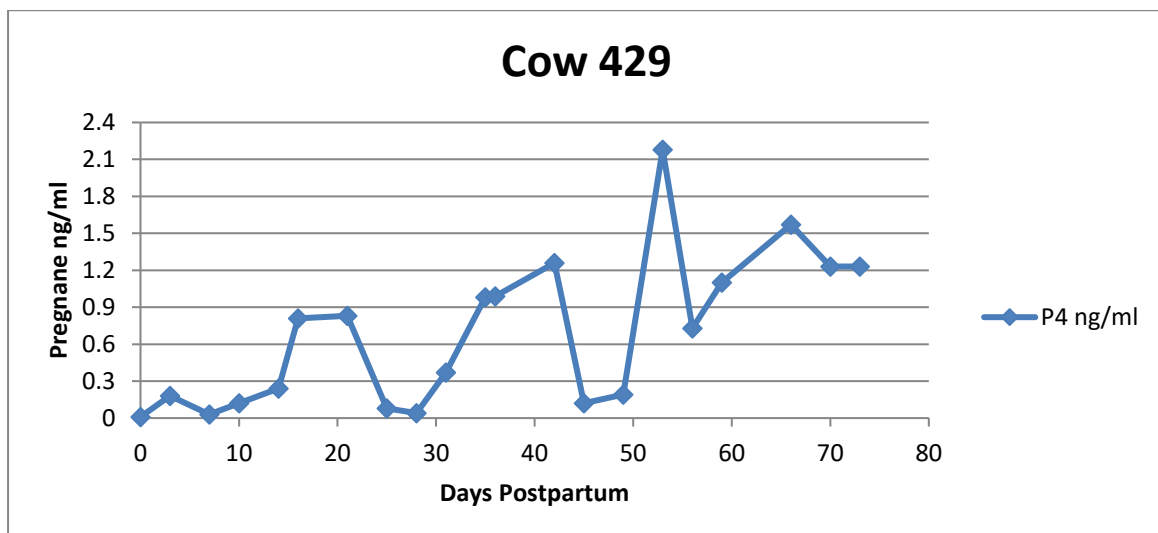
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 54 postpartum; 0.68ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(61 - 54) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(68 - 64) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(54 - 1.75) = 52.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



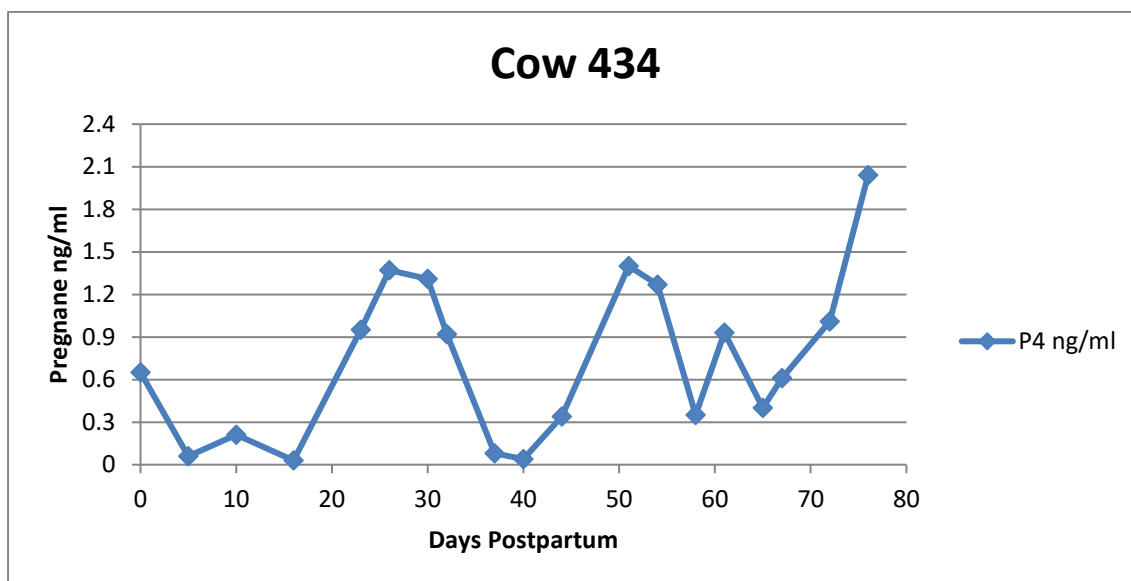
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 25; 0.33ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(46 - 25) + 3.5 = 24.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(54 - 49) + 3.5 = 8.5$ days
- CLA : $(T1 - 1.75)$; $(25 - 1.75) = 23.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(67 - 60) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



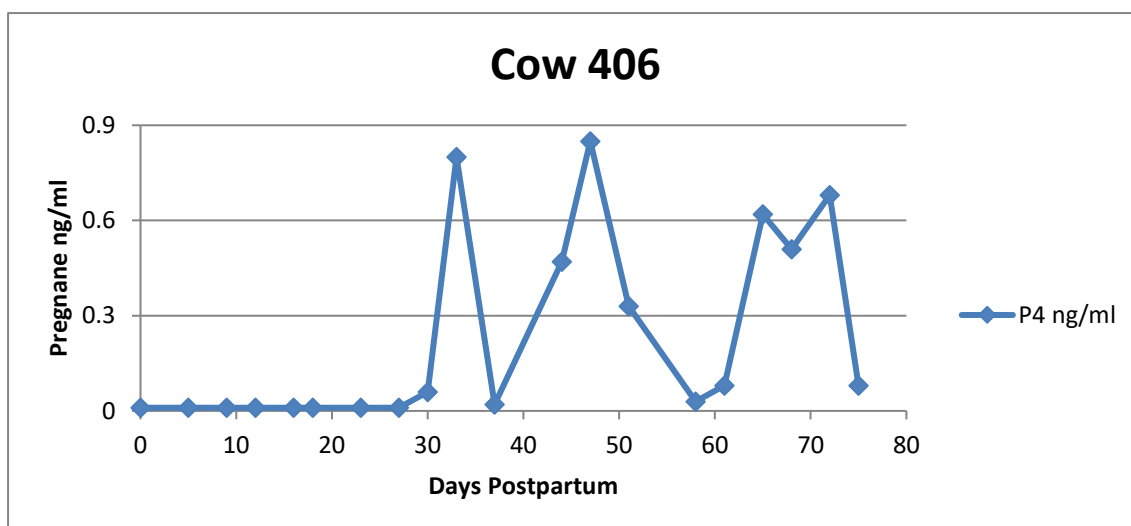
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 16; 0.81ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(21 - 16) + 3.5 = 8.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(28 - 25) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(16 - 1.75) = 14.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(42 - 31) + 3.5 = 14.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(49 - 45) + 3.5 = 7.5$ days



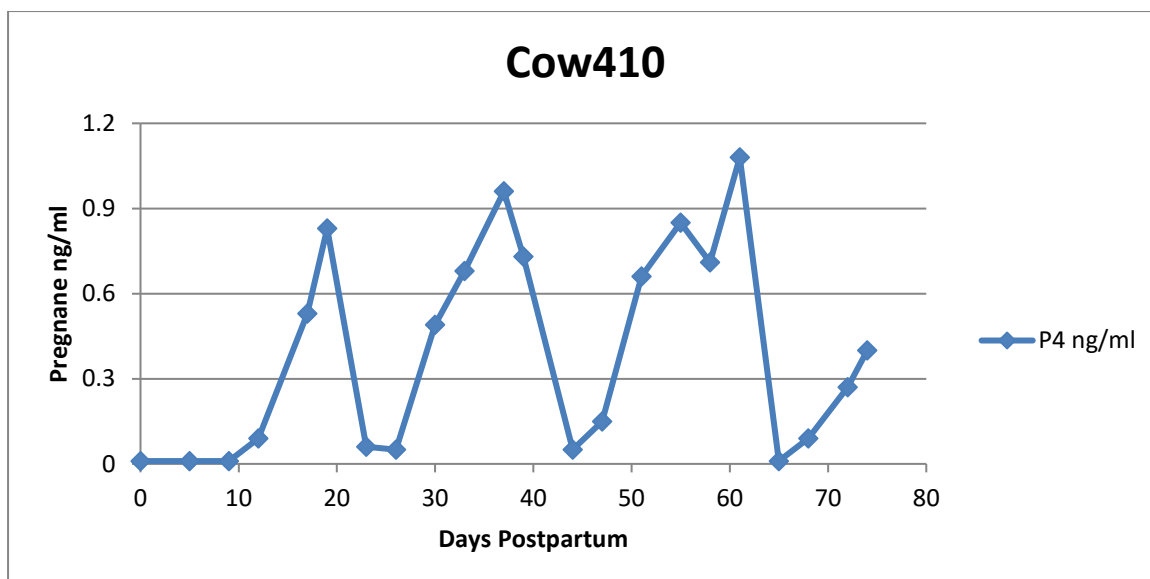
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 0.95ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(32 - 23) + 3.5 = 12.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(40 - 37) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(76 - 44) + 3.5 = 35.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$



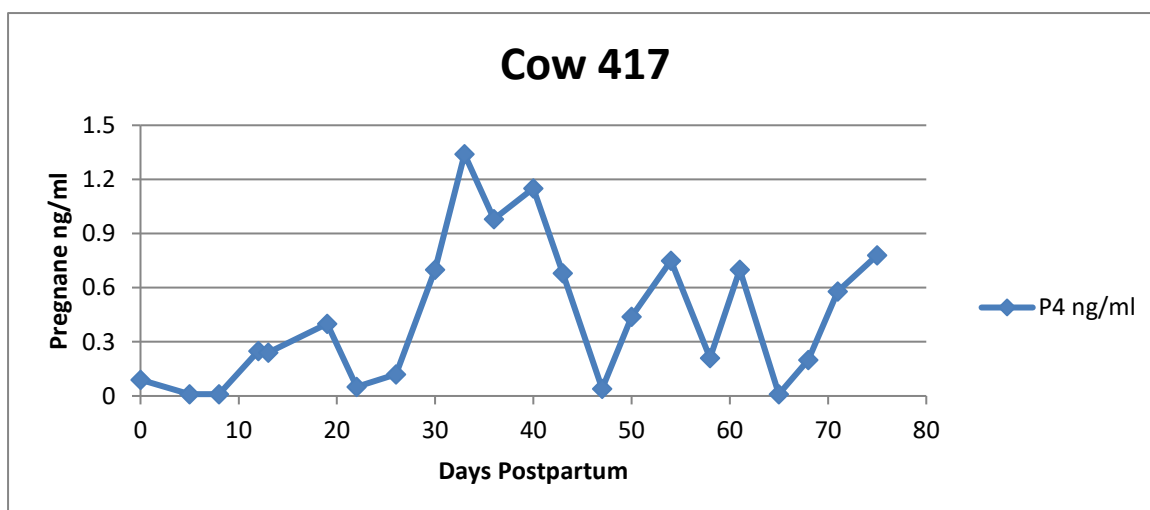
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 33; 1.39ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(33 - 1.75) = 31.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(51 - 44) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(61 - 58) + 3.5 = 6.5$ days



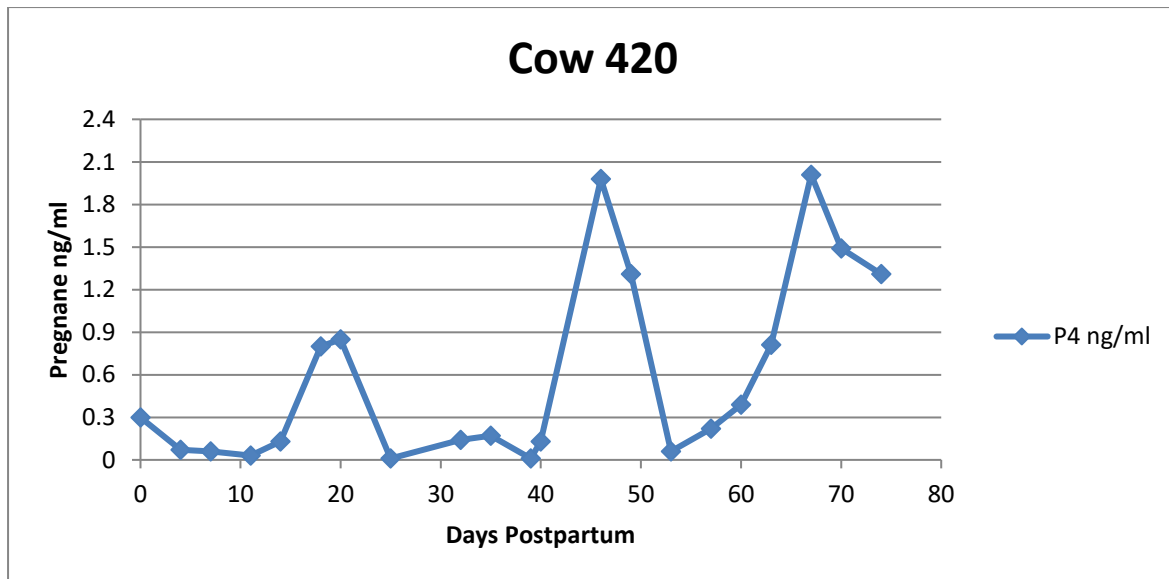
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 17; 0.53ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(19 - 17) + 3.5 = 5.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(26 - 23) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(17 - 1.75) = 15.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(39 - 30) + 3.5 = 12.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(47 - 44) + 3.5 = 6.5$ days



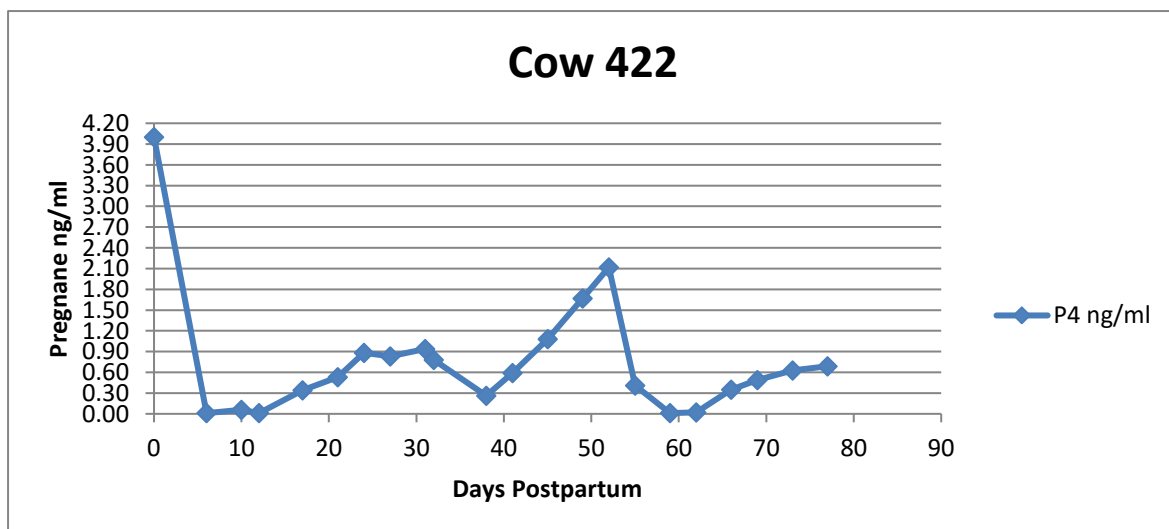
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 19; 0.40ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(26 - 22) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(19 - 1.75) = 17.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(43 - 30) + 3.5 = 16.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



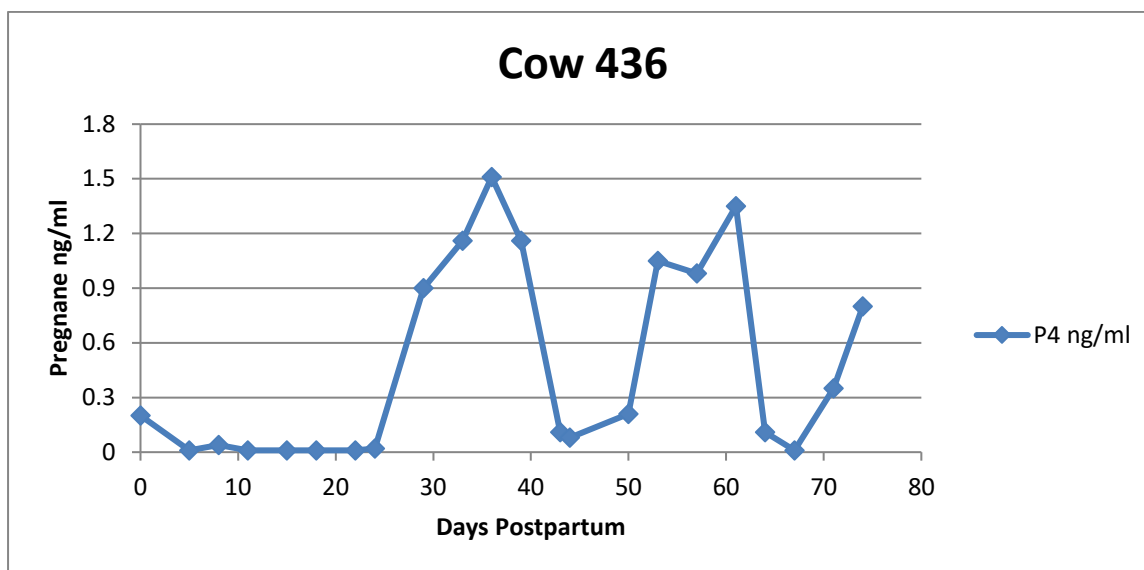
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.80ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(20 - 18) + 3.5 = 5.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(40 - 25) + 3.5 = 18.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(49 - 46) + 3.5 = 6.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(57 - 53) + 3.5 = 7.5$ days



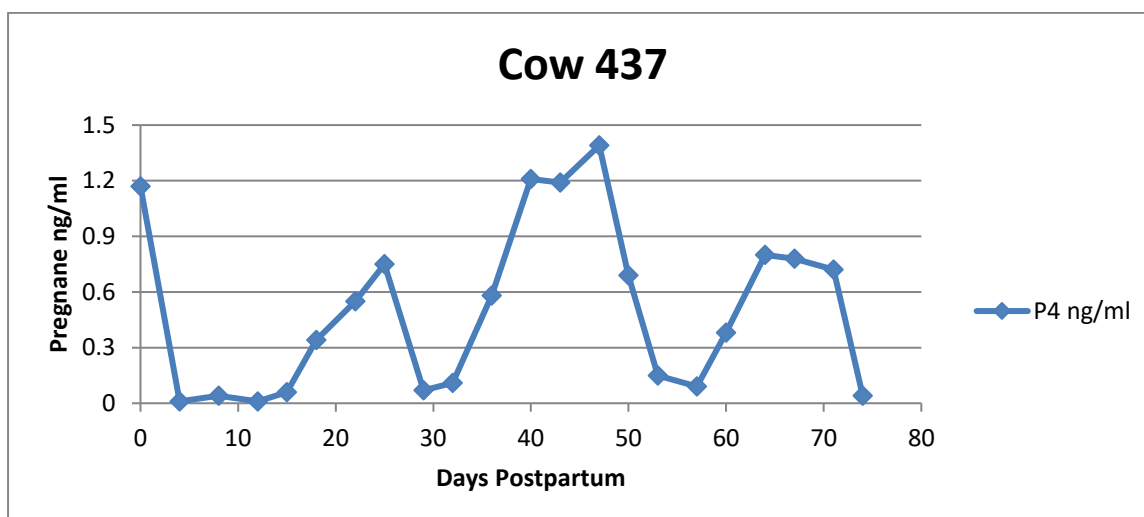
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 17; 0.34ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(32 - 17) + 3.5 = 18.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(17 - 1.75) = 15.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(55 - 41) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(62 - 59) + 3.5 = 6.5$ days



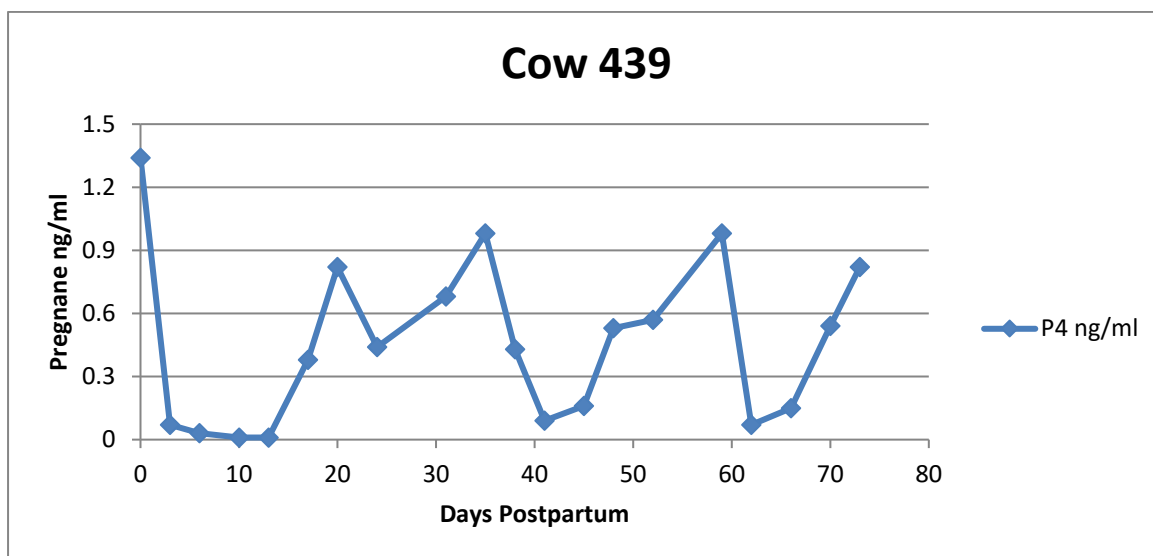
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 29; 0.90ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(39 - 29) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(50 - 43) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(29 - 1.75) = 27.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(61 - 53) + 3.5 = 11.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(67 - 64) + 3.5 = 6.5$ days



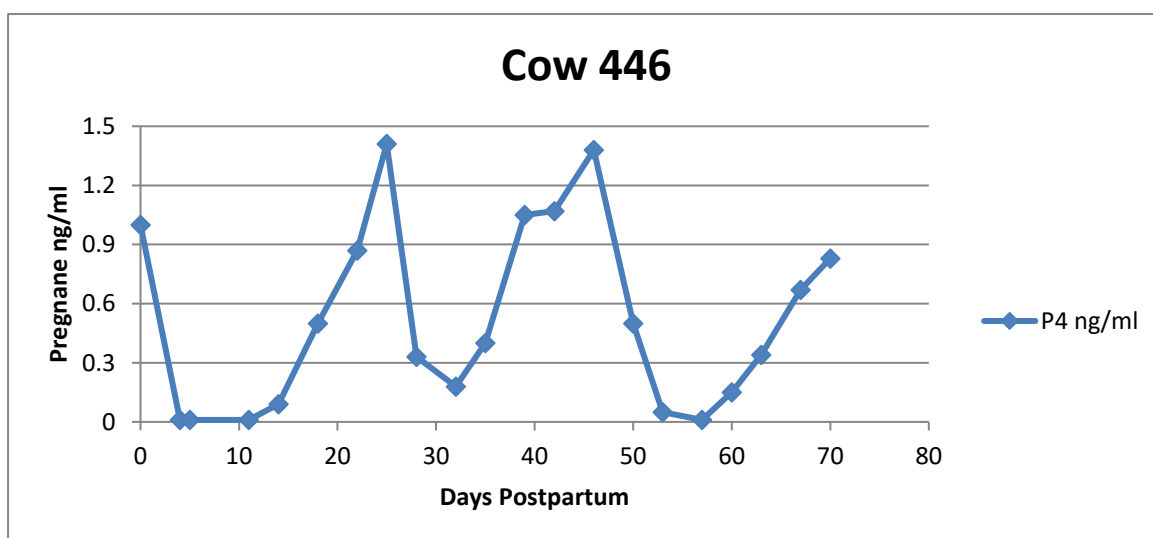
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.34ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(25 - 18) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(32 - 29) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(50 - 36) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(57 - 53) + 3.5 = 7.5$ days



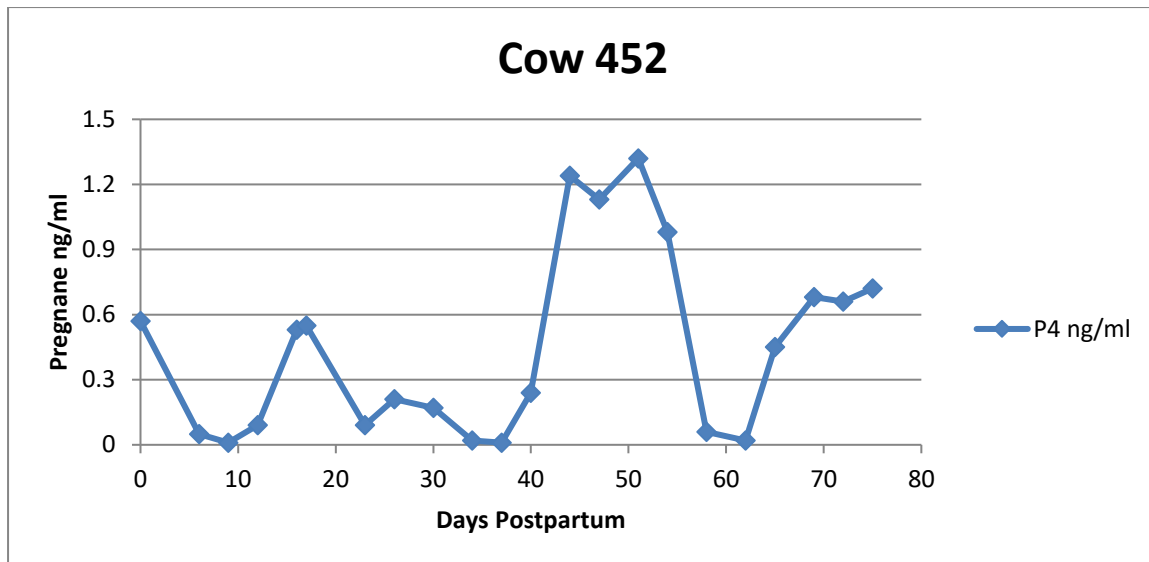
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 17; 0.38ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(38 - 17) + 3.5 = 24.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(45 - 41) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(17 - 1.75) = 15.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(59 - 48) + 3.5 = 14.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(66 - 62) + 3.5 = 7.5$ days



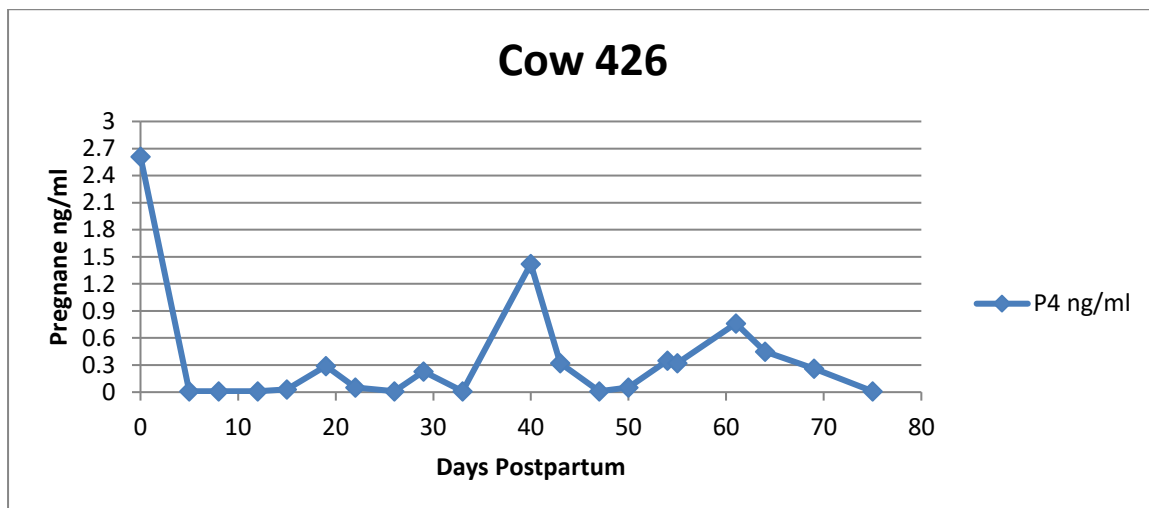
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.50ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(28 - 18) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(35 - 32) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(50 - 35) + 3.5 = 18.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(60 - 53) + 3.5 = 10.5$ days



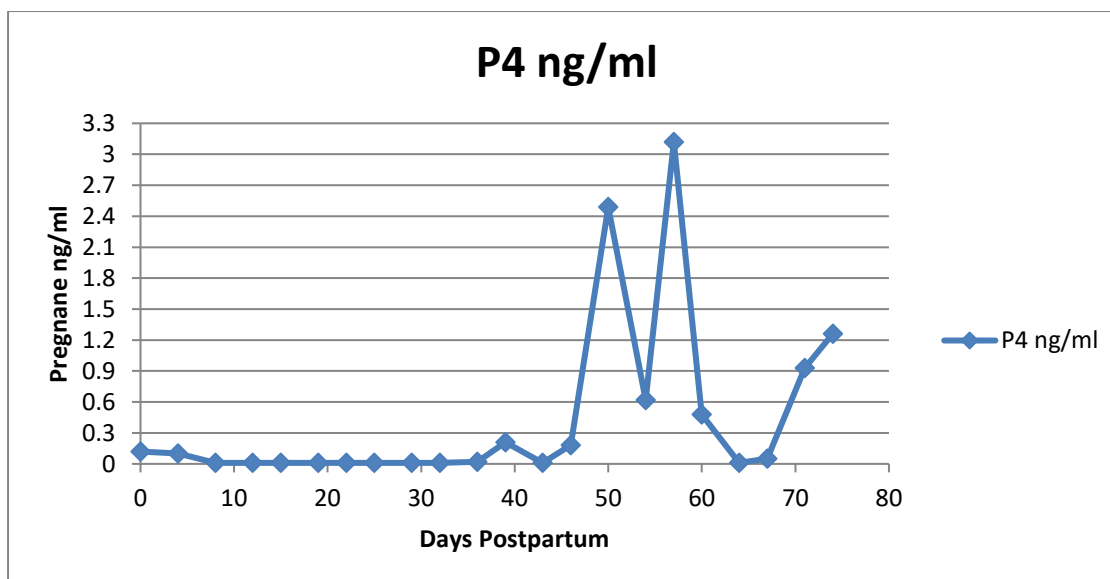
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 16; 0.53ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(17 - 16) + 3.5 = 4.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(40 - 23) + 3.5 = 20.5$ days
- CLA : $(T1 - 1.75)$; $(16 - 1.75) = 14.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(54 - 44) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(62 - 58) + 3.5 = 7.5$ days



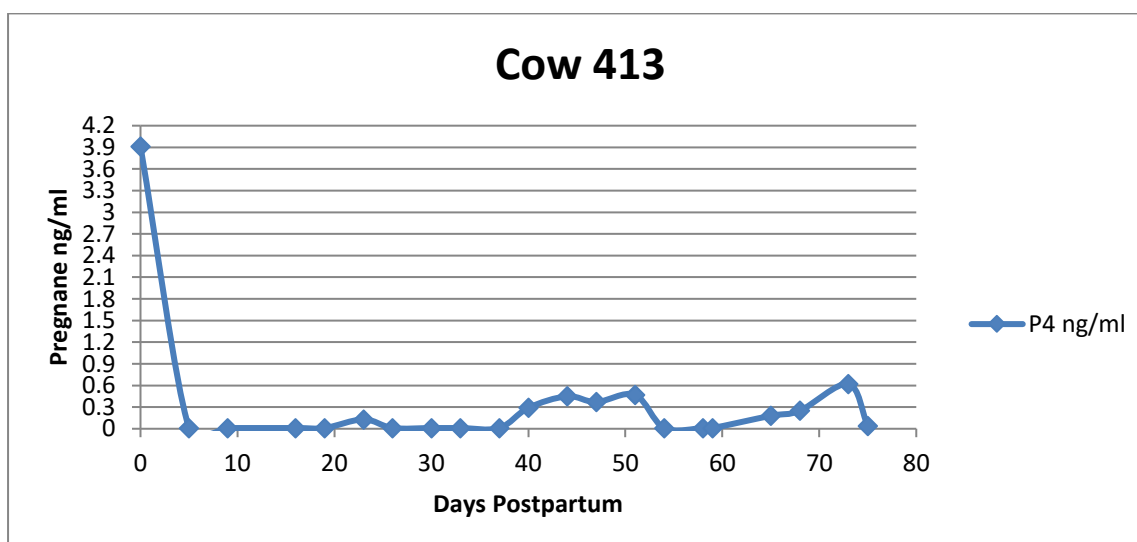
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 40; 1.42ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(43 - 40) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(50 - 47) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(40 - 1.75) = 38.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(64 - 54) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(75 - 69) + 3.5 = 9.5$ days



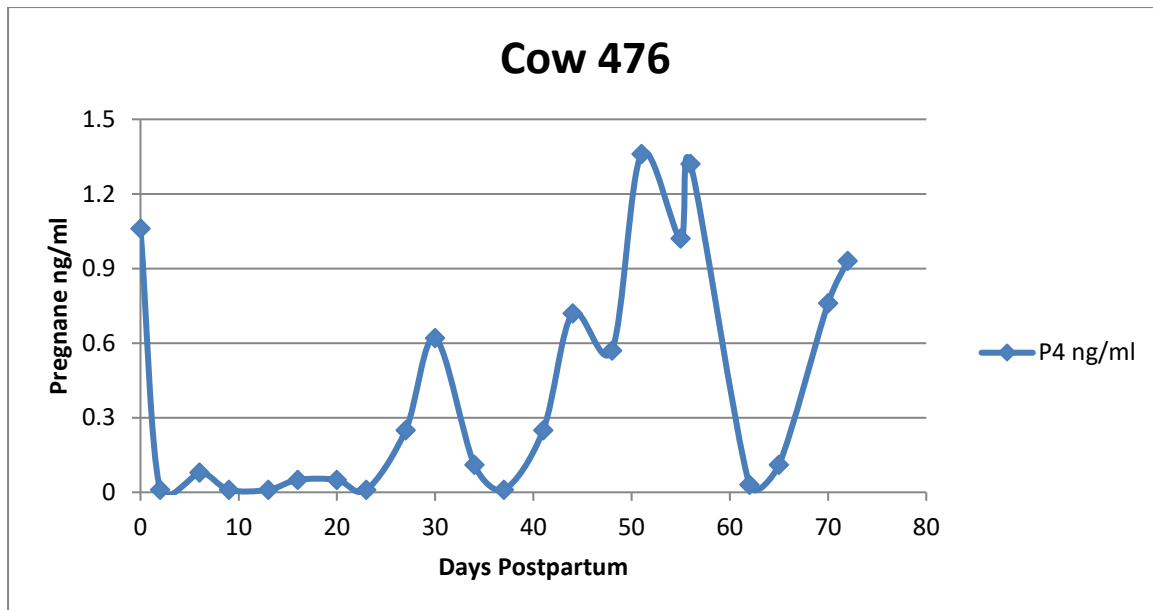
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 50 postpartum; 2.49ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(60 - 50) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(67 - 64) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(50 - 1.75) = 48.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



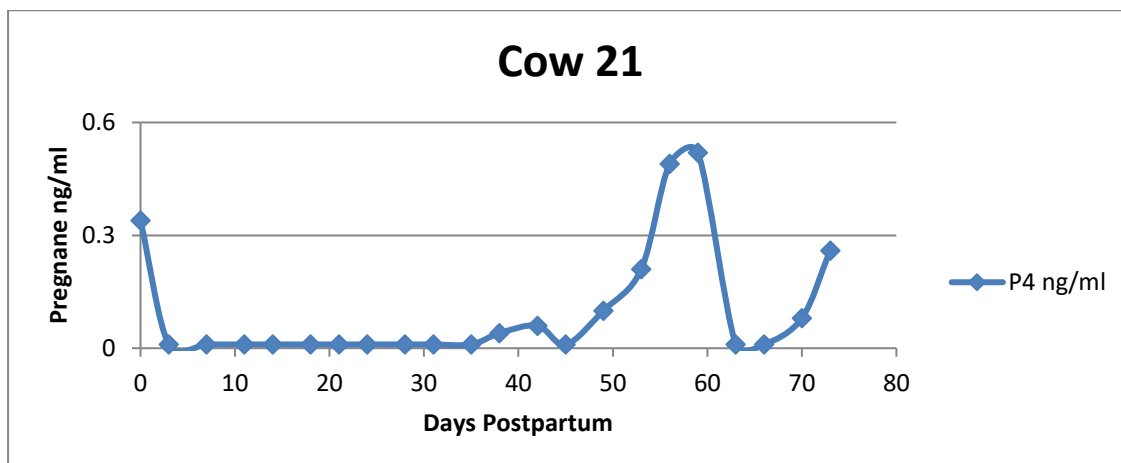
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 44; 0.45ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(51 - 44) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(68 - 54) + 3.5 = 17.5$ days
- CLA : $(T1 - 1.75)$; $(44 - 1.75) = 42.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



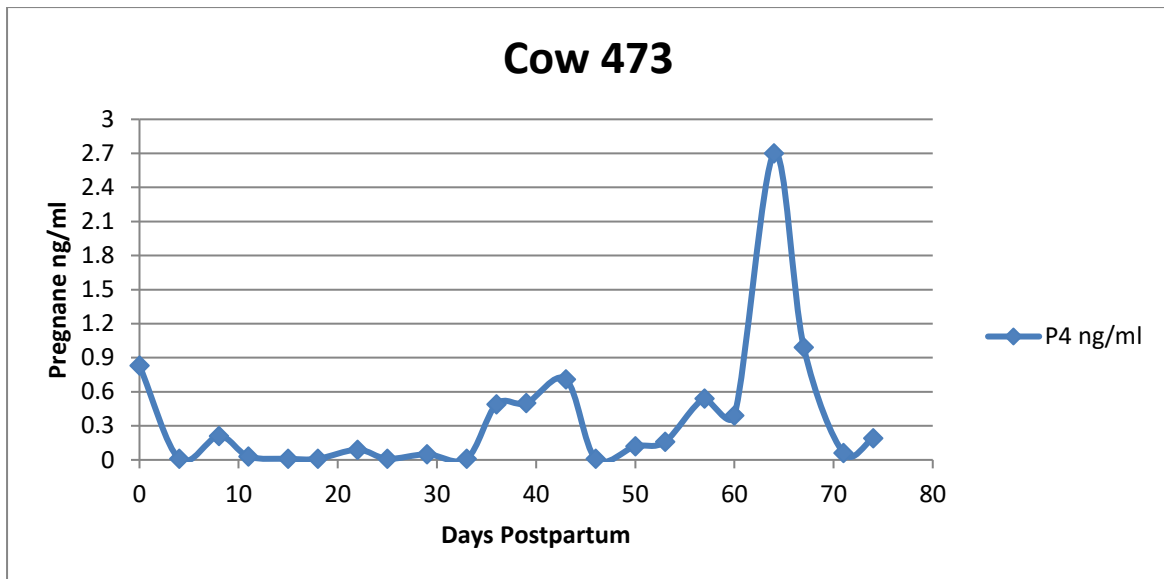
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 30; 0.62ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(41 - 34) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(30 - 1.75) = 28.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(56 - 44) + 3.5 = 15.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(65 - 62) + 3.5 = 6.5$ days



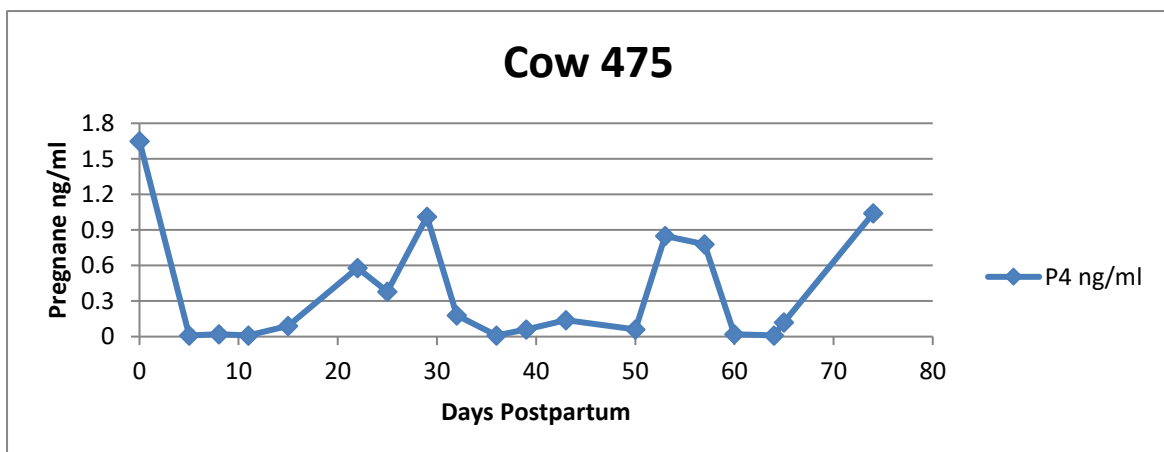
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 56 postpartum; 0.49ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(59 - 56) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(73 - 63) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(56 - 1.75) = 54.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



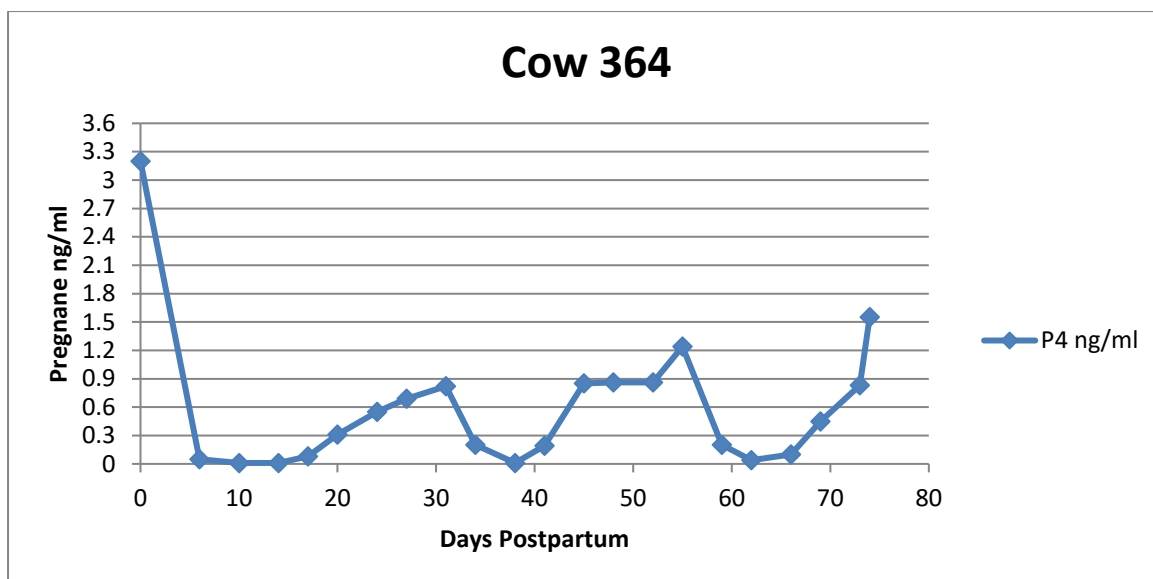
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 36; 0.49ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(43 - 36) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(53 - 46) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(36 - 1.75) = 34.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(67 - 57) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(74 - 71) + 3.5 = 6.5$ days



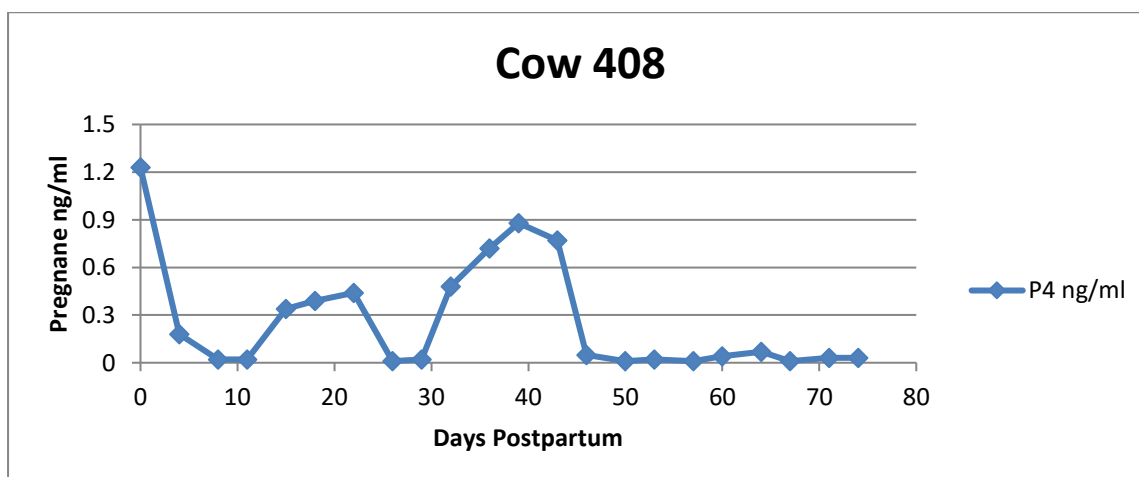
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 22; 0.58ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 22) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(50 - 32) + 3.5 = 21.5$ days
- CLA : $(T1 - 1.75)$; $(22 - 1.75) = 20.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(57 - 53) + 3.5 = 7.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(65 - 60) + 3.5 = 8.5$ days



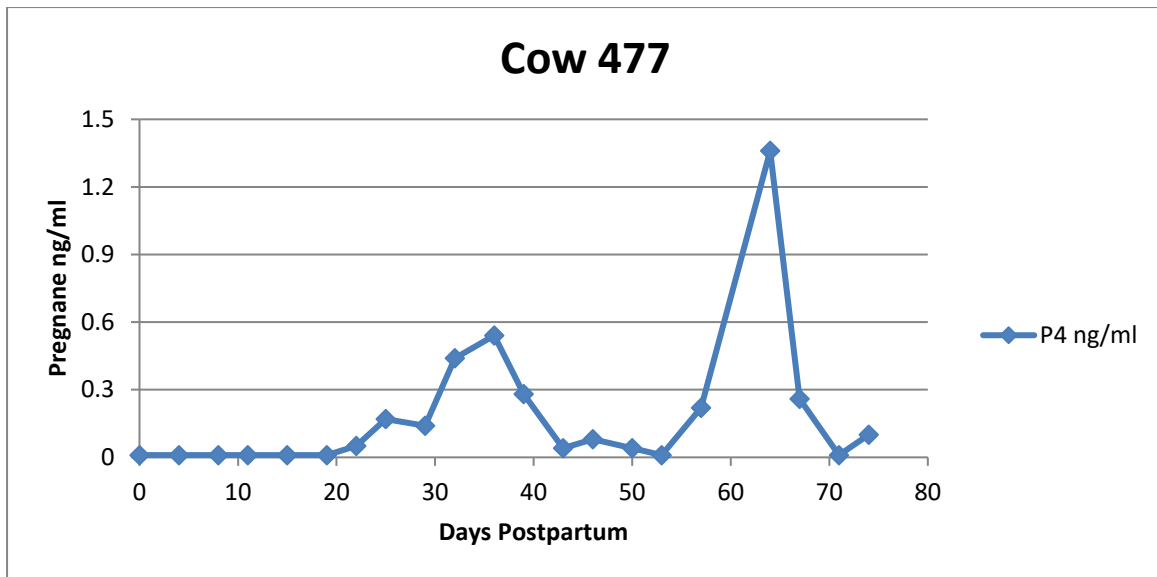
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 20; 0.31ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(31 - 20) + 3.5 = 14.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(41 - 34) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(20 - 1.75) = 18.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(55 - 45) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(66 - 59) + 3.5 = 10.5$ days



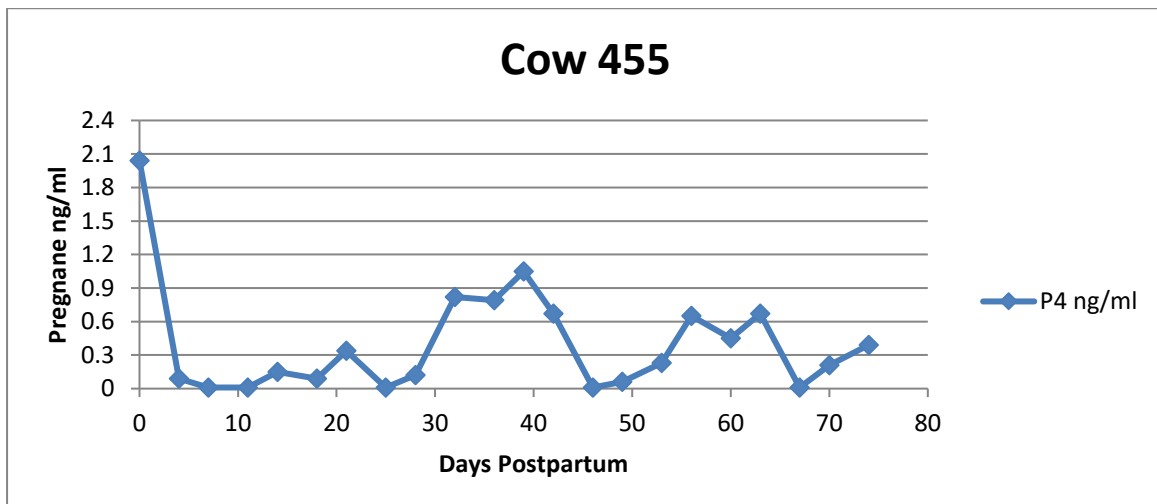
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 15; 0.34ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(22 - 15) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(29 - 26) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(15 - 1.75) = 13.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(43 - 32) + 3.5 = 14.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(74 - 46) + 3.5 = 31.5$ days



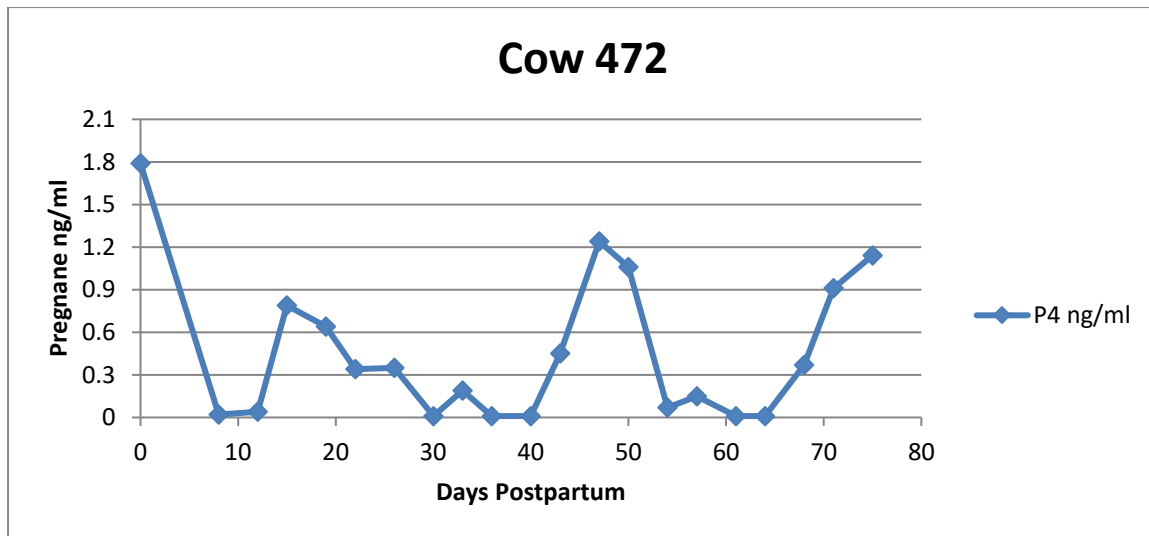
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 32; 0.44ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(36 - 32) + 3.5 = 7.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(57 - 39) + 3.5 = 21.5$ days
- CLA : $(T1 - 1.75)$; $(32 - 1.75) = 30.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(74 - 67) + 3.5 = 10.5$ days



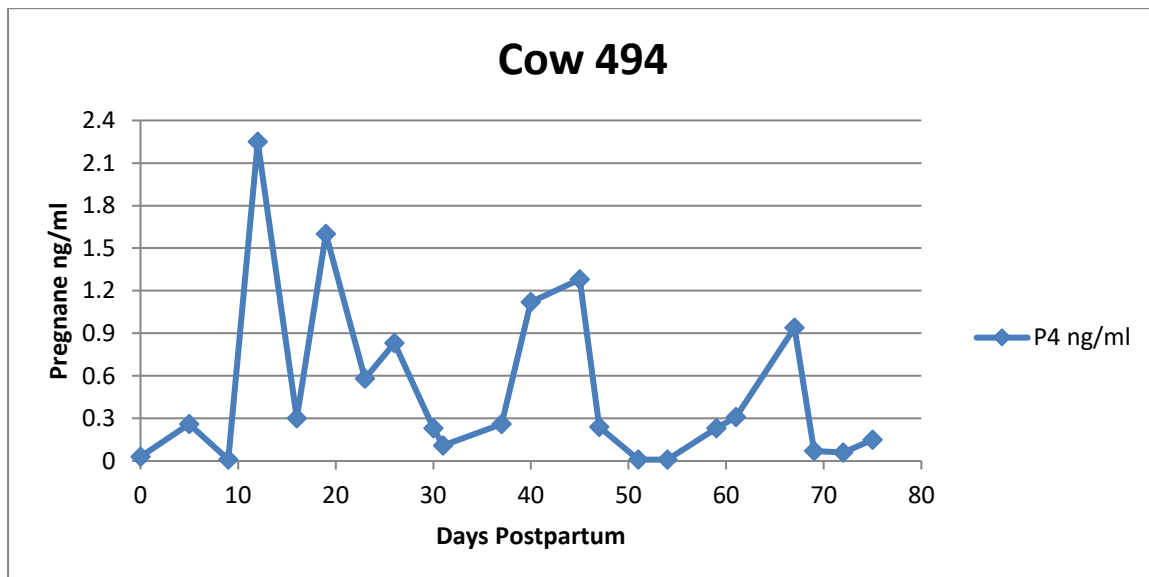
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 21; 0.34ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(28 - 25) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(21 - 1.75) = 19.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(42 - 32) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(53 - 46) + 3.5 = 10.5$ days



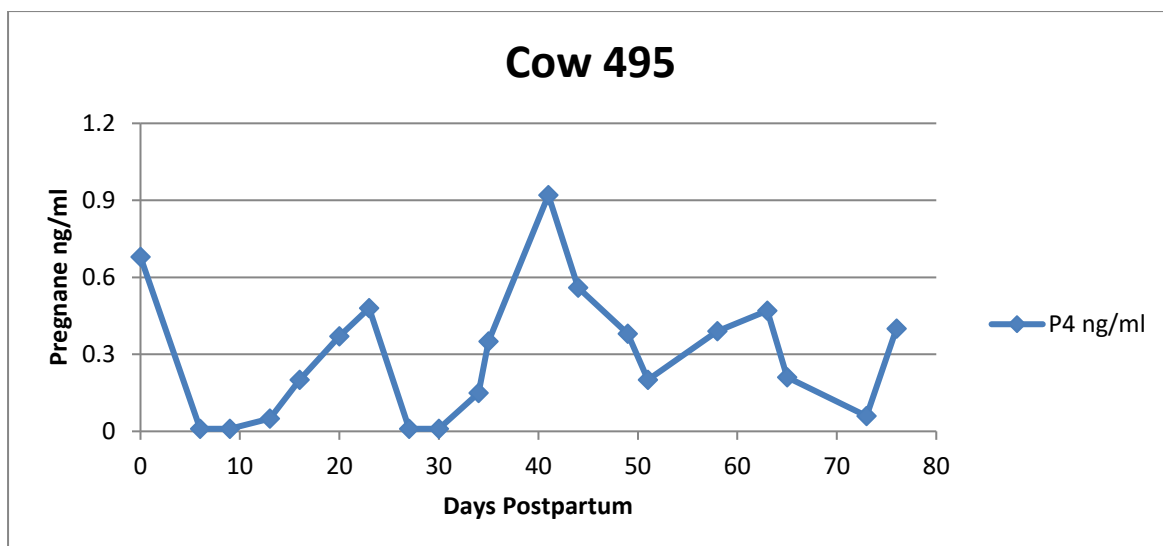
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 15; 0.79ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(26 - 15) + 3.5 = 14.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(40 - 30) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(15 - 1.75) = 13.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(50 - 43) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(64 - 54) + 3.5 = 13.5$ days



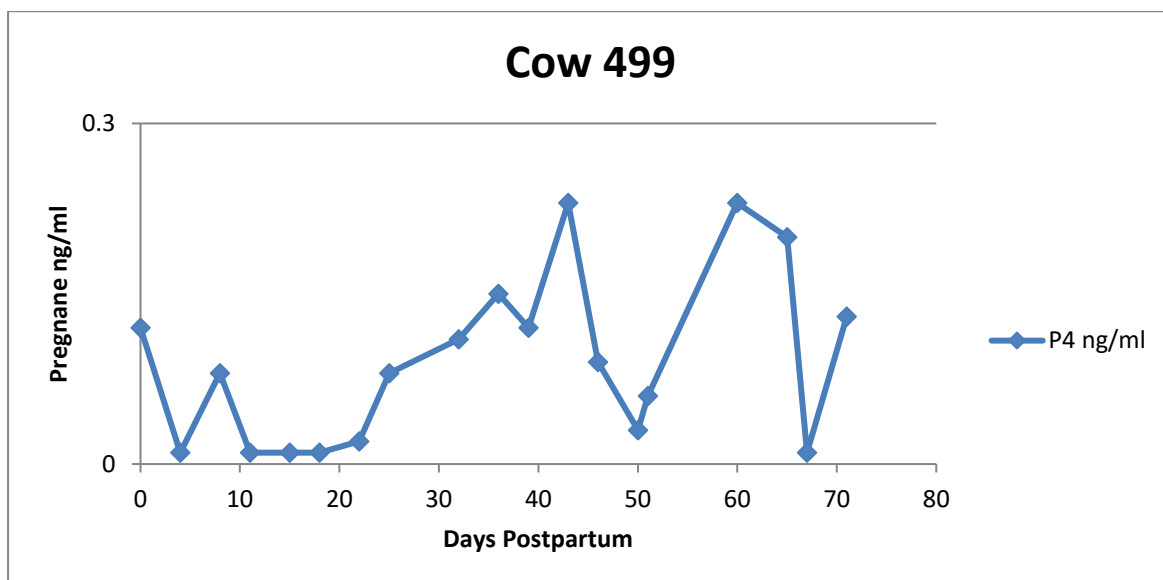
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 12; 2.25ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(26 - 12) + 3.5 = 17.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(37 - 30) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(12 - 1.75) = 10.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(45 - 40) + 3.5 = 8.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(59 - 47) + 3.5 = 15.5$ days



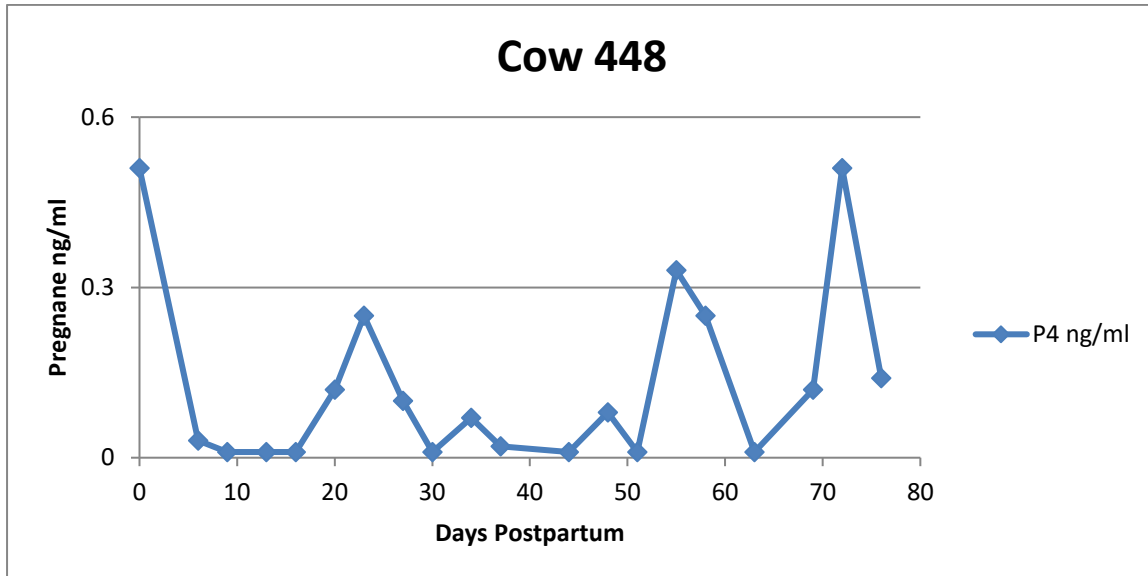
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 20; 0.37ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(23 - 20) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(34 - 27) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(20 - 1.75) = 18.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(49 - 35) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



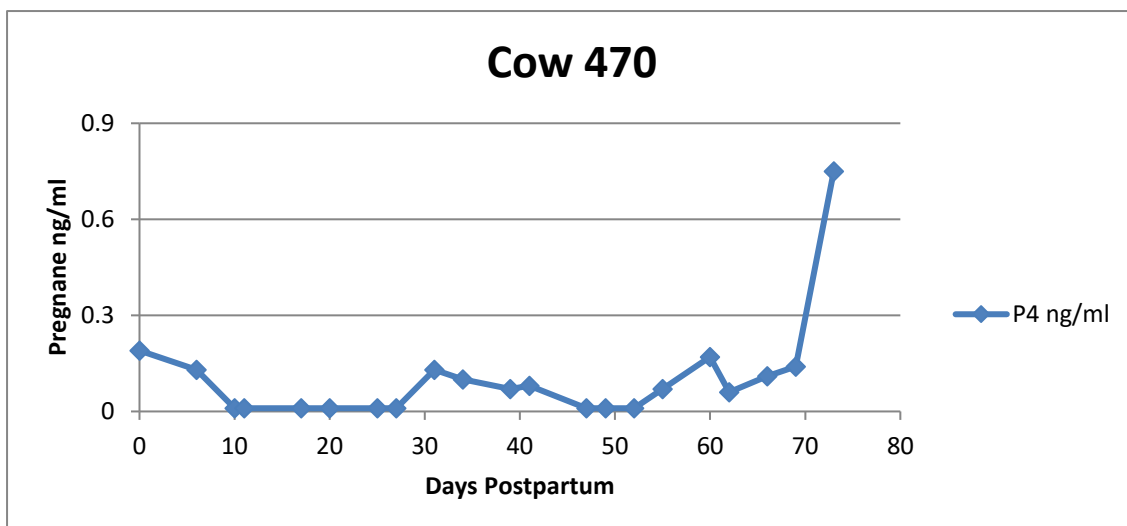
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- 1st LP : $(T2 - T1 + 3.5)$;
- 1st ILI : $(T6 - T5 + 3.5)$;
- CLA : $(T1 - 1.75)$;
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



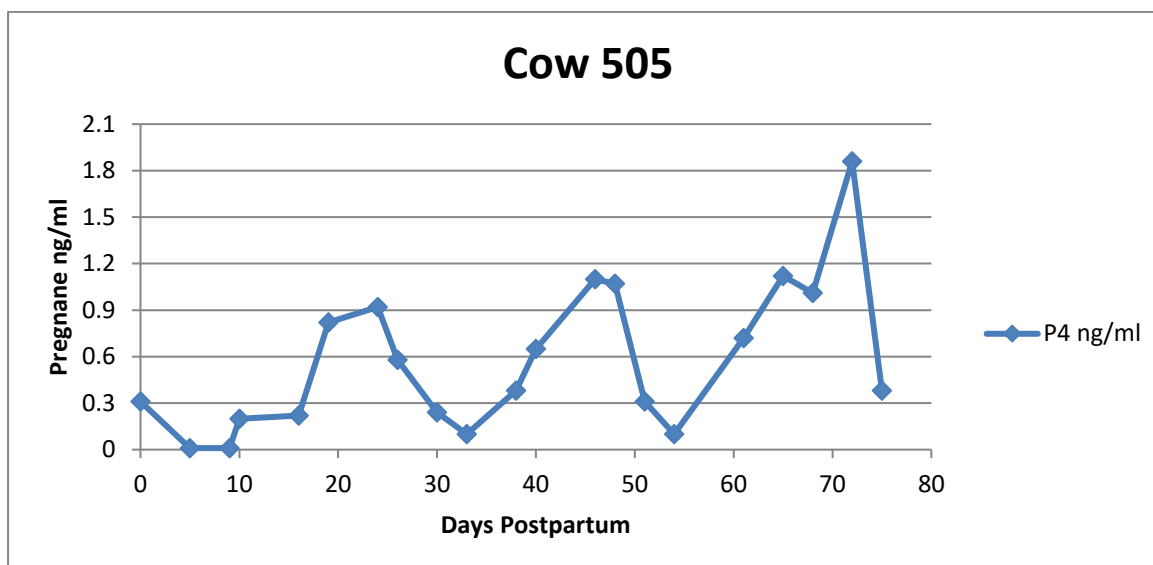
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 55 postpartum; 0.33ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(69 - 58) + 3.5 = 14.5$ days
- CLA : $(T1 - 1.75)$; $(55 - 1.75) = 53.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$



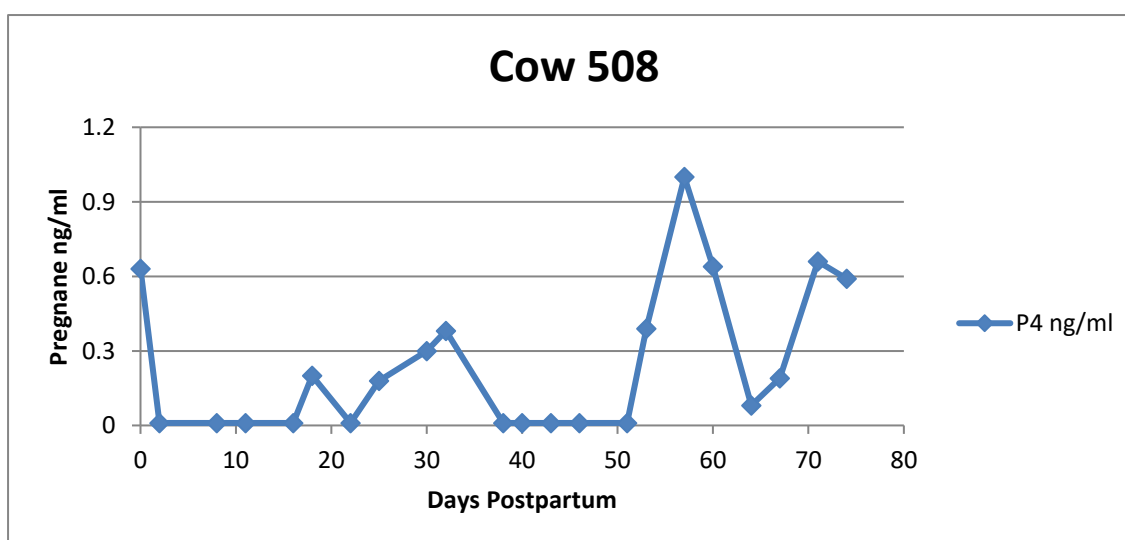
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 73 postpartum; 0.75ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$
- CLA : $(T1 - 1.75)$; $(73 - 1.75) = 71.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



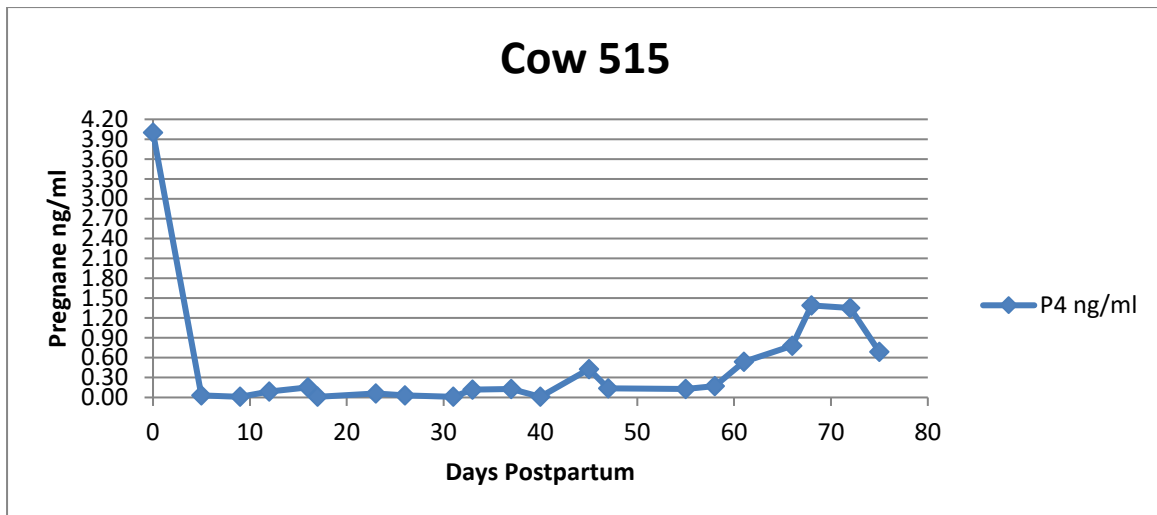
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 19; 0.82ng/ml).
- 1st LP : (T2 – T1 + 3.5); (26 – 19) + 3.5 = 10.5 days
- 1st ILI : (T6 – T5 + 3.5); (33 – 30) + 3.5 = 6.5 days
- CLA : (T1 – 1.75); (19 – 1.75) = 17.25 days
- 2nd LP : (T2 – T1 + 3.5); (51 – 38) + 3.5 = 16.5 days
- 2nd ILI : (T6 – T5 + 3.5) = 3.5 days
-



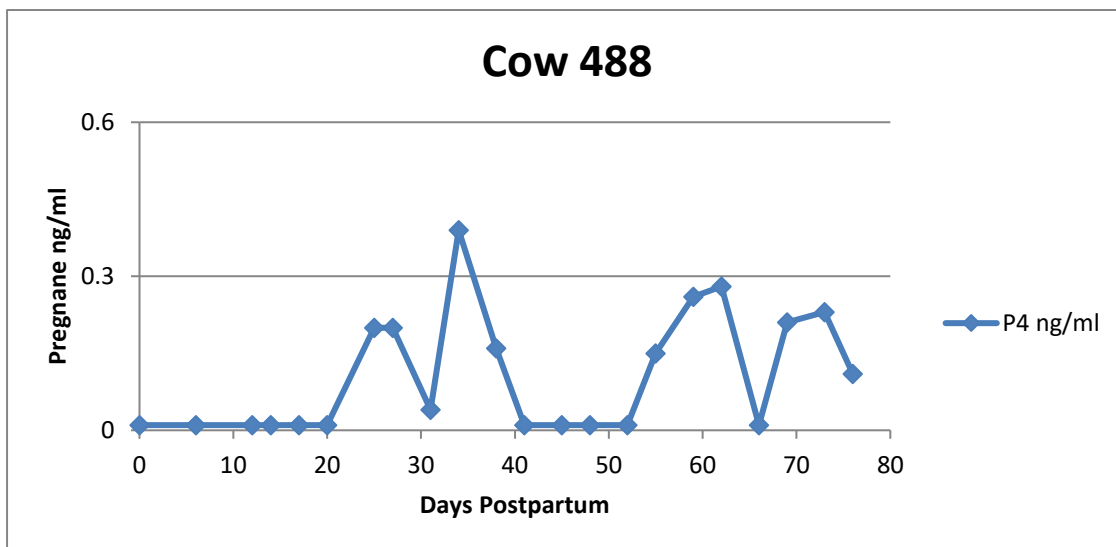
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 30; 0.30ng/ml).
- 1st LP : (T2 – T1 + 3.5); (32 – 30) + 3.5 = 5.5 days
- 1st ILI : (T6 – T5 + 3.5); (51 – 38) + 3.5 = 16.5 days
- CLA : (T1 – 1.75); (30 – 1.75) = 28.25 days
- 2nd LP : (T2 – T1 + 3.5); (60 – 53) + 3.5 = 10.5 days
- 2nd ILI : (T6 – T5 + 3.5); (67 – 64) + 3.5 = 6.5 days



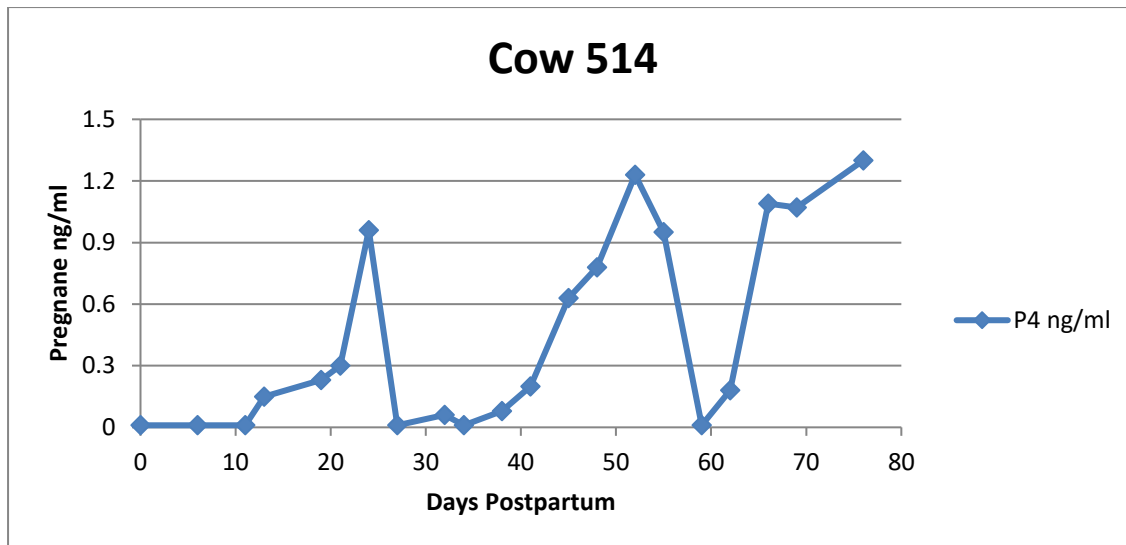
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 45; 0.43ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(58 - 47) + 3.5 = 14.5$ days
- CLA : $(T1 - 1.75)$; $(45 - 1.75) = 43.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



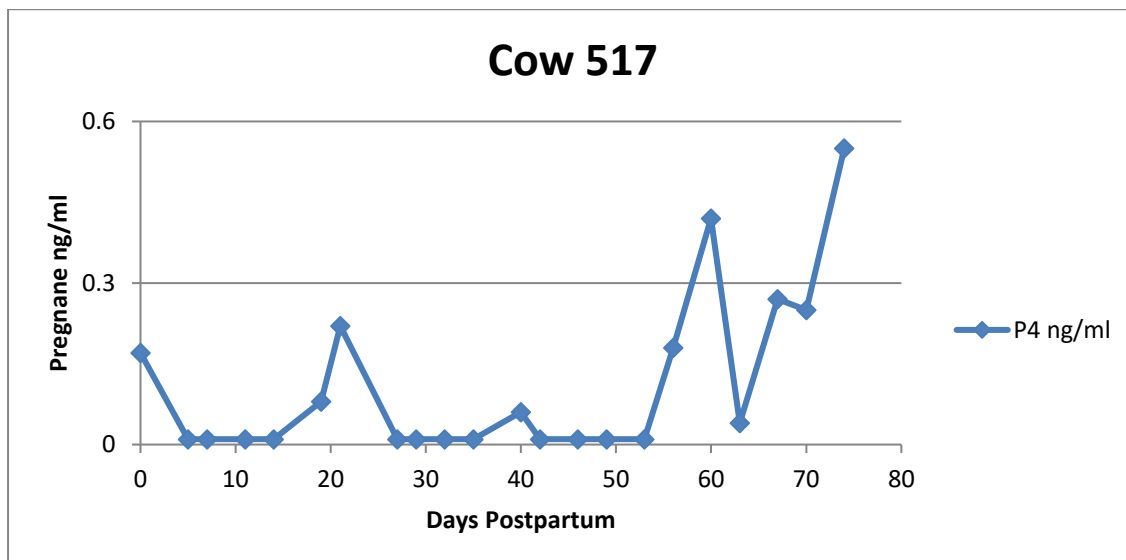
DOV2

- First rise of pregnane (>0.3ng/ml) occurred at day 34 postpartum; 0.39ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(76 - 38) + 3.5 = 41.5$ days
- CLA : $(T1 - 1.75)$; $(34 - 1.75) = 32.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



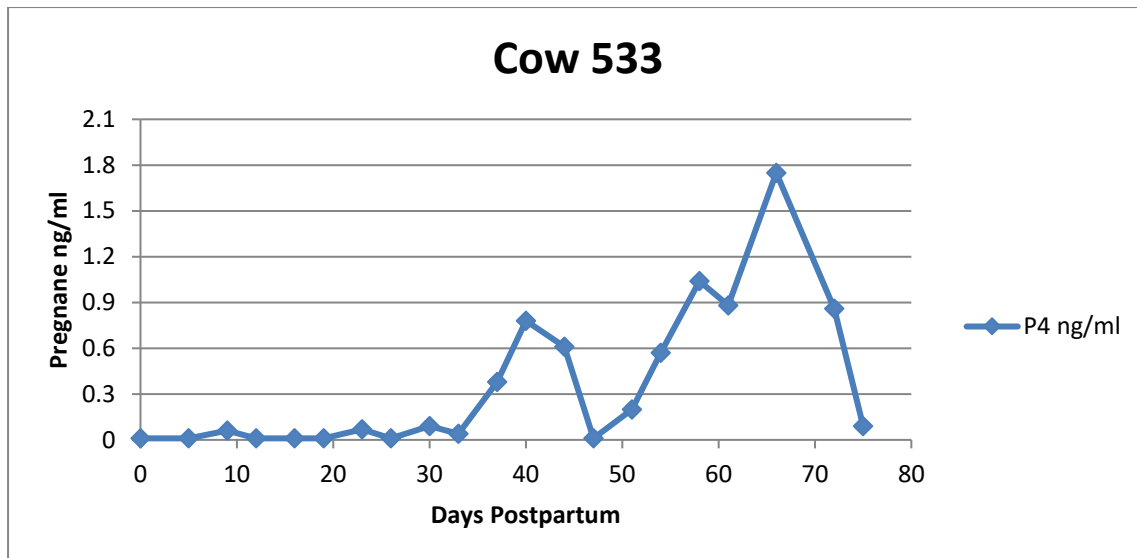
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 21; 0.30ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(24 - 21) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(41 - 27) + 3.5 = 17.5$ days
- CLA : $(T1 - 1.75)$; $(21 - 1.75) = 19.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(55 - 45) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(62 - 59) + 3.5 = 6.5$ days
-



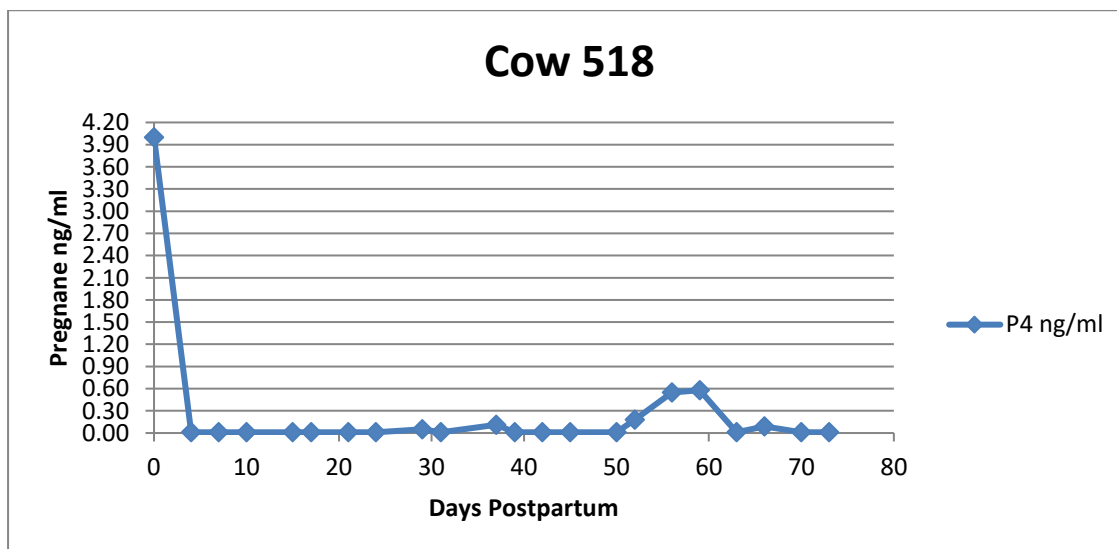
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 60 postpartum; 0.42ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(70 - 63) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(60 - 1.75) = 58.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



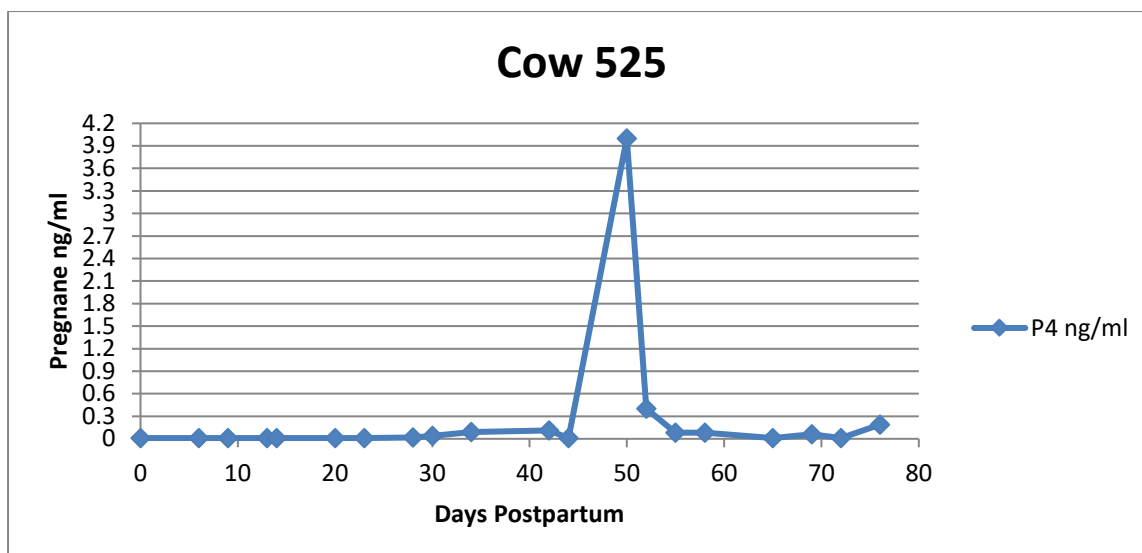
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 37; 0.38ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(44 - 37) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(51 - 47) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(37 - 1.75) = 35.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(72 - 54) + 3.5 = 21.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$;



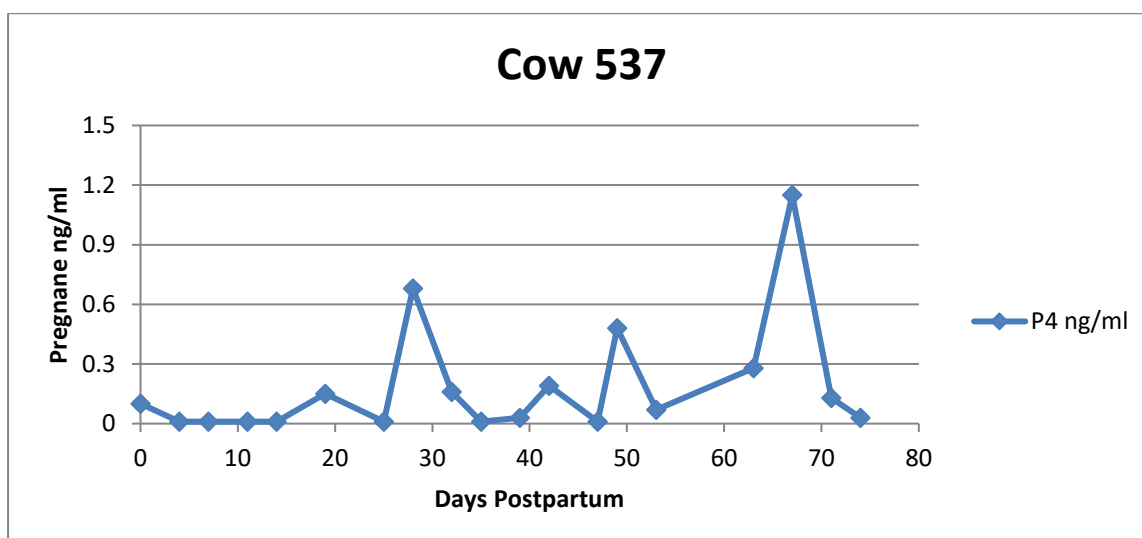
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 56 postpartum; 0.55ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(59 - 56) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(73 - 63) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(56 - 1.75) = 54.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



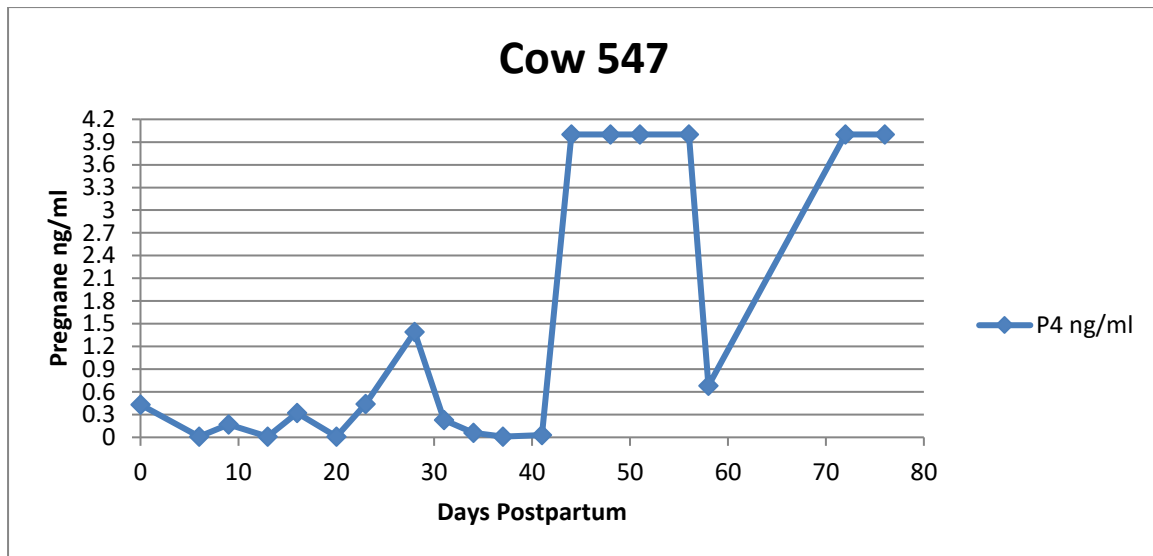
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 50; 4.00ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(52 - 50) + 3.5 = 5.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(76 - 55) + 3.5 = 24.5$ days
- CLA : $(T1 - 1.75)$; $(50 - 1.75) = 48.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



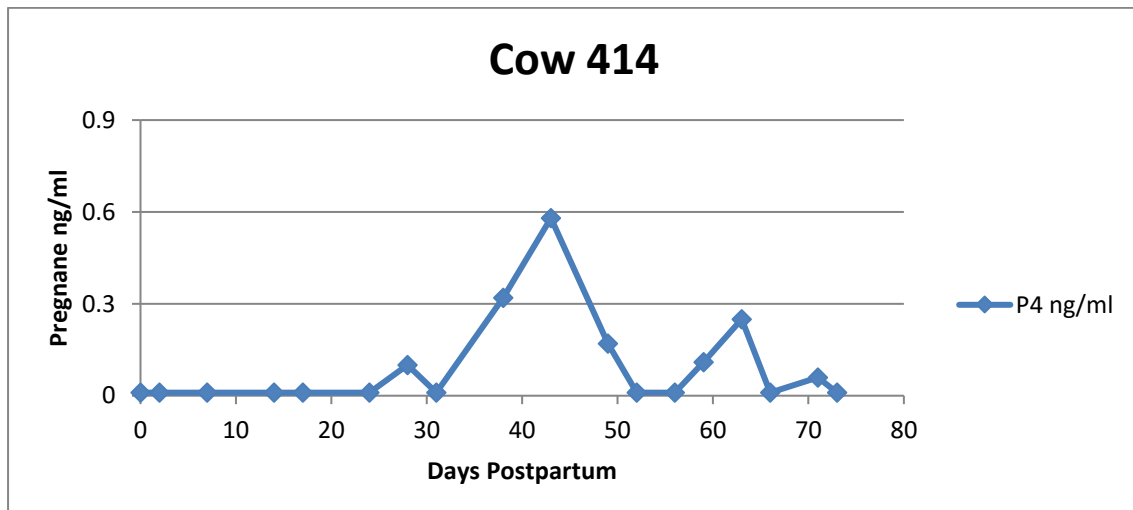
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 28; 0.68ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(47 - 32) + 3.5 = 18.5$ days
- CLA : $(T1 - 1.75)$; $(28 - 1.75) = 26.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(63 - 53) + 3.5 = 13.5$ days



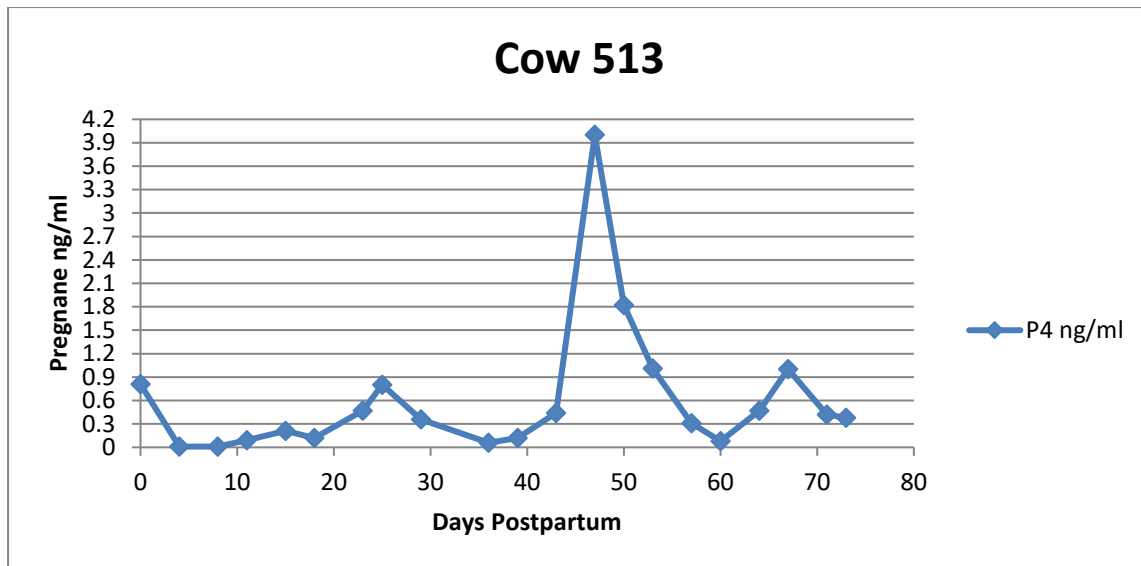
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 16; 0.32ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(16 - 1.75) = 14.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(28 - 23) + 3.5 = 8.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(41 - 31) + 3.5 = 13.5$ days



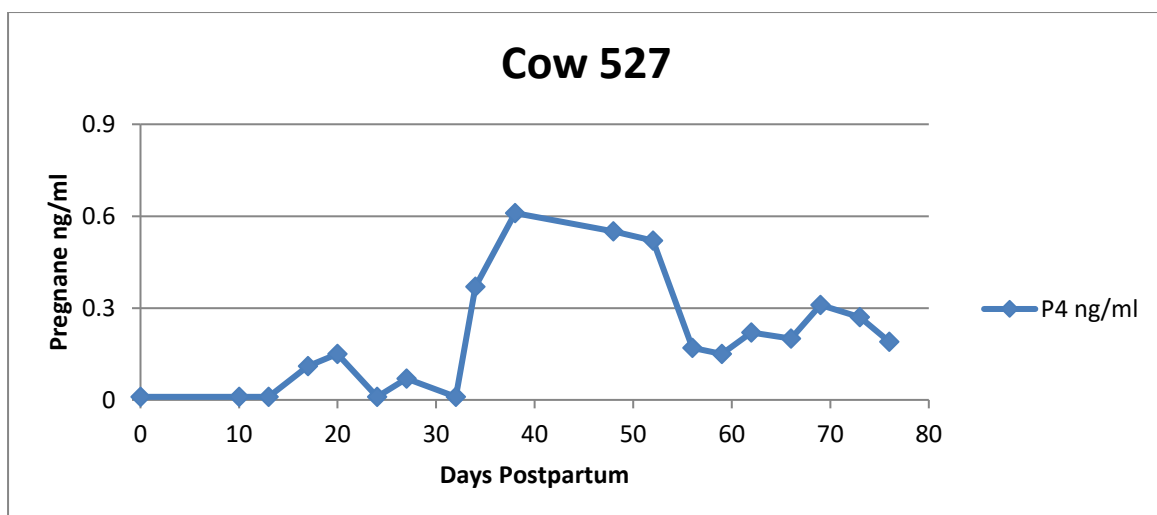
DOV2

- First rise of pregnane (>0.3ng/ml) occurred at day 38 postpartum; 0.32ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(43 - 38) + 3.5 = 8.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(73 - 49) + 3.5 = 27.5$ days
- CLA : $(T1 - 1.75)$; $(38 - 1.75) = 36.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



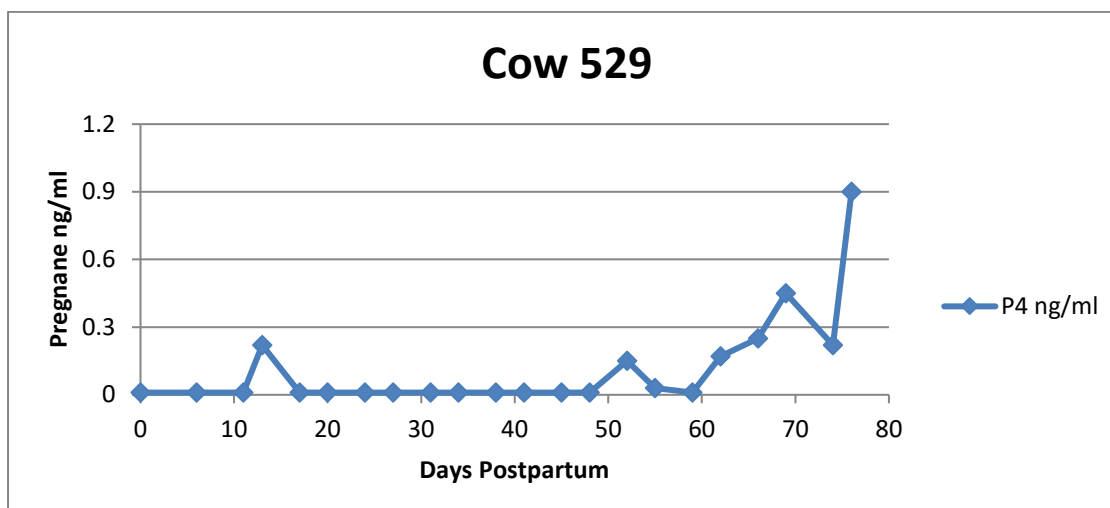
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 0.47ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 23) + 3.5 = 9.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(39 - 36) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(57 - 43) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



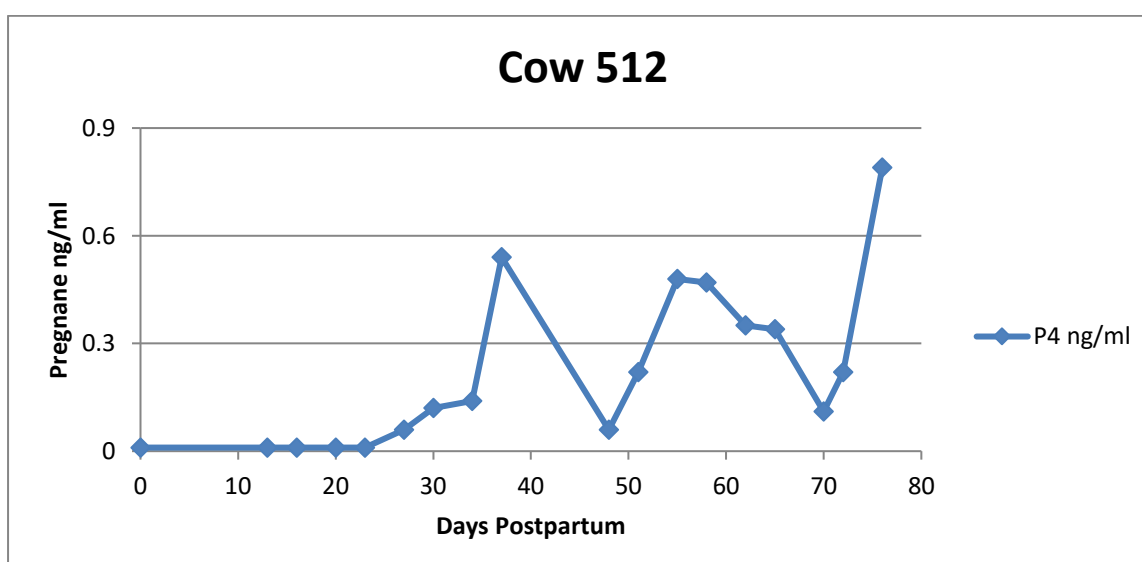
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 34; 0.37ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(52 - 34) + 3.5 = 21.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(66 - 56) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(34 - 1.75) = 32.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$



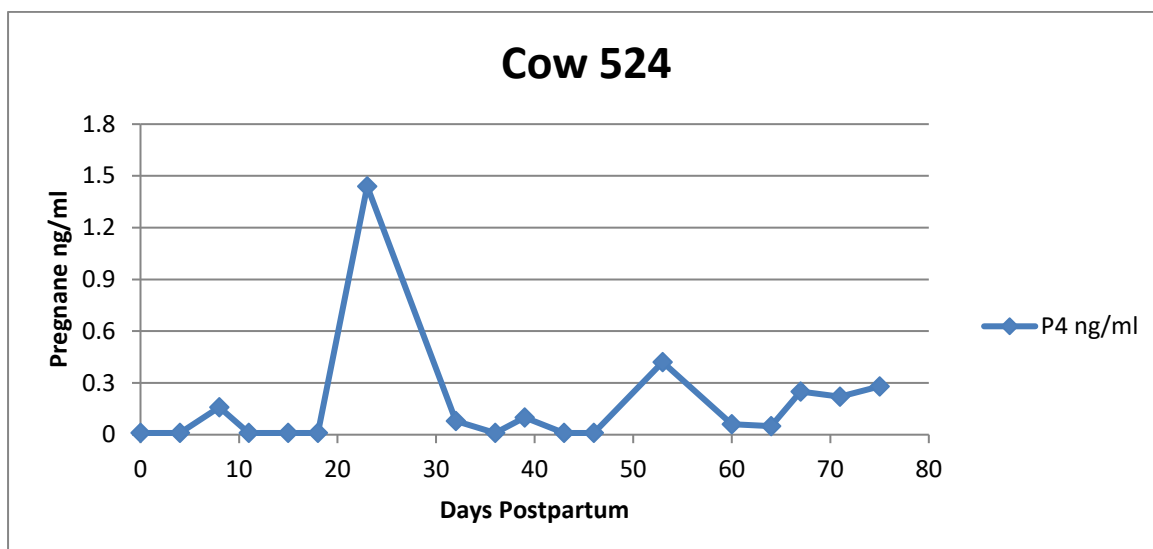
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 69 postpartum; 0.45ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75); (69 - 1.75) = 67.25$ days
- 2nd LP : $(T2 - T1 + 3.5);$
- 2nd ILI : $(T6 - T5 + 3.5);$



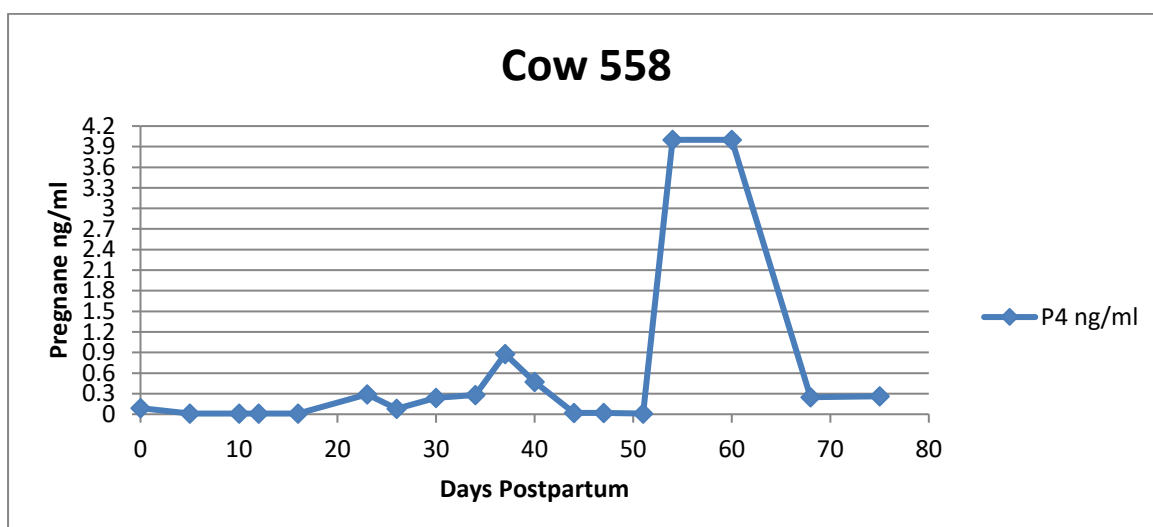
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 37; 0.54ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5); (51 - 48) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75); (37 - 1.75) = 35.25$ days
- 2nd LP : $(T2 - T1 + 3.5); (65 - 55) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5); (72 - 70) + 3.5 = 5.5$ days



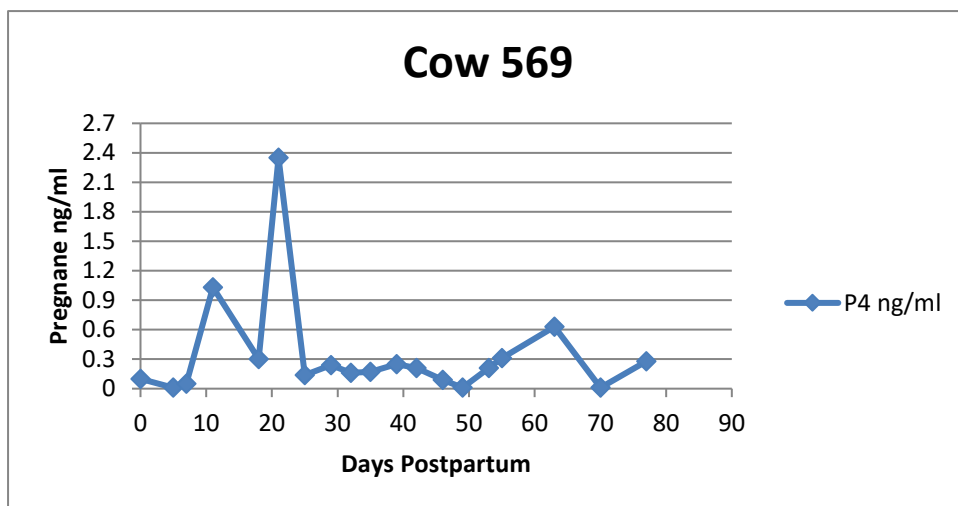
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 1.44ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(46 - 32) + 3.5 = 17.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(75 - 60) + 3.5 = 18.5$ days



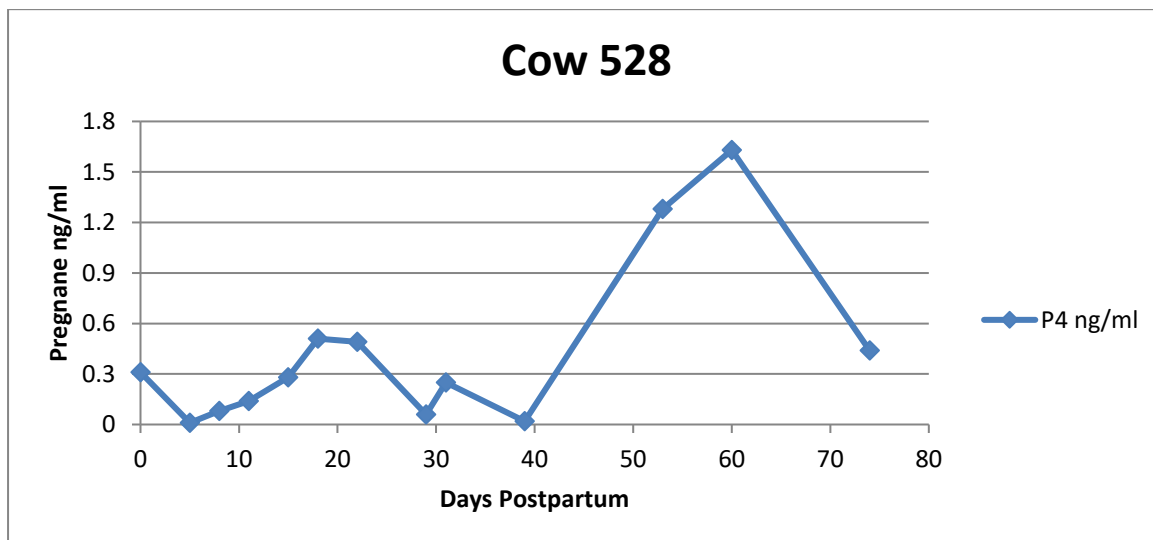
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 37; 0.88ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(40 - 37) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(51 - 44) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(37 - 1.75) = 35.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(60 - 54) + 3.5 = 9.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(75 - 68) + 3.5 = 10.5$ days



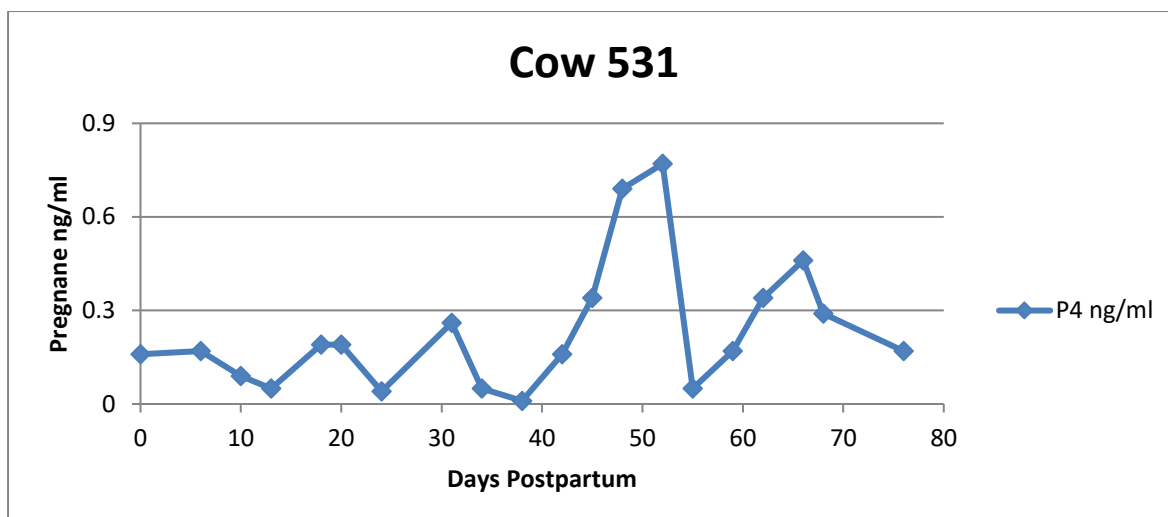
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 11; 1.03ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(21 - 11) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(53 - 25) + 3.5 = 31.5$ days
- CLA : $(T1 - 1.75)$; $(11 - 1.75) = 9.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(63 - 55) + 3.5 = 11.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(77 - 70) + 3.5 = 10.5$ days



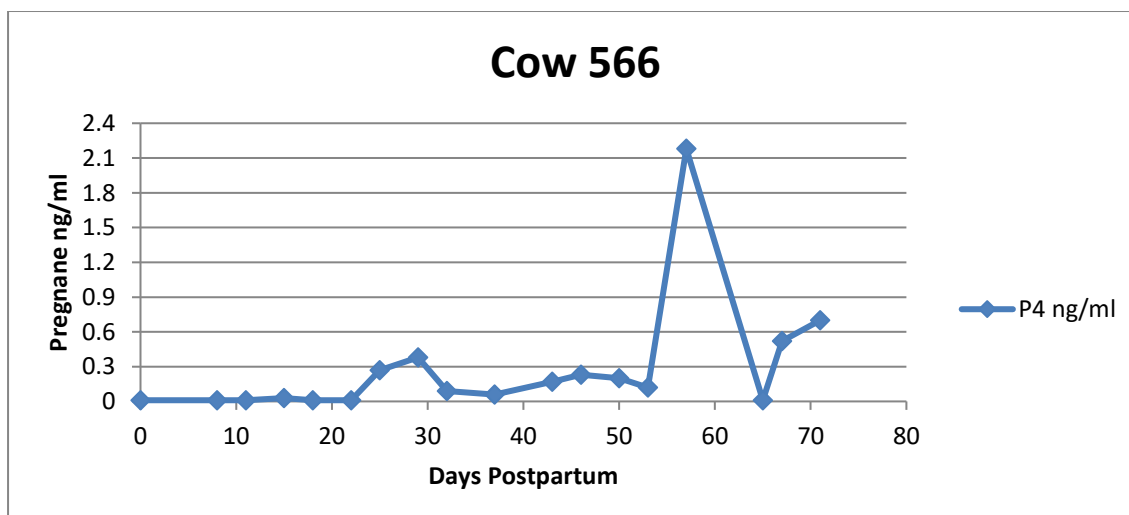
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.51ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(22 - 18) + 3.5 = 7.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(39 - 29) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(74 - 53) + 3.5 = 24.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$;



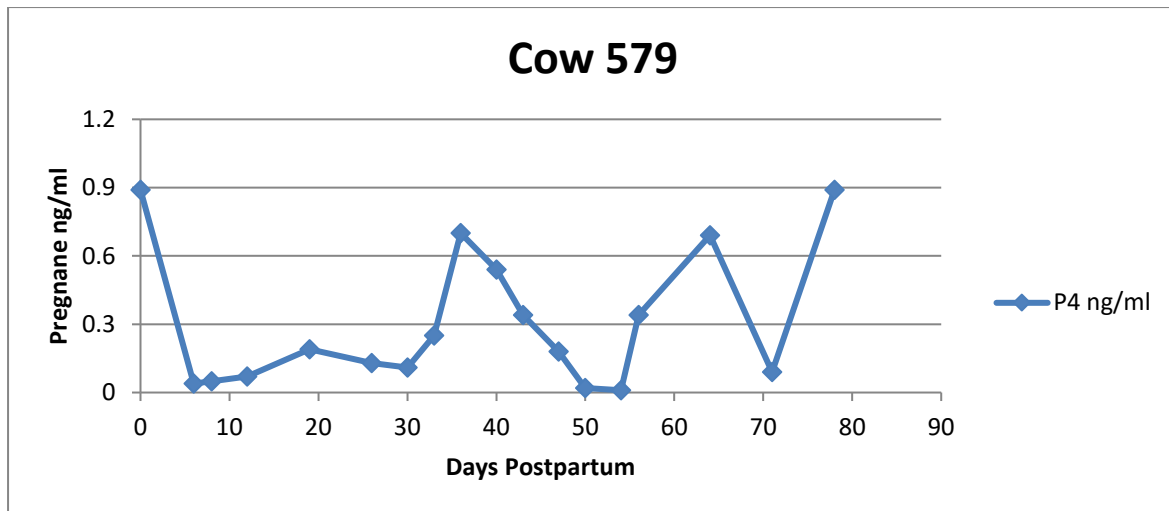
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 45; 0.34ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(52 - 45) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(59 - 55) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(45 - 1.75) = 43.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(66 - 62) + 3.5 = 7.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(76 - 68) + 3.5 = 11.5$ days



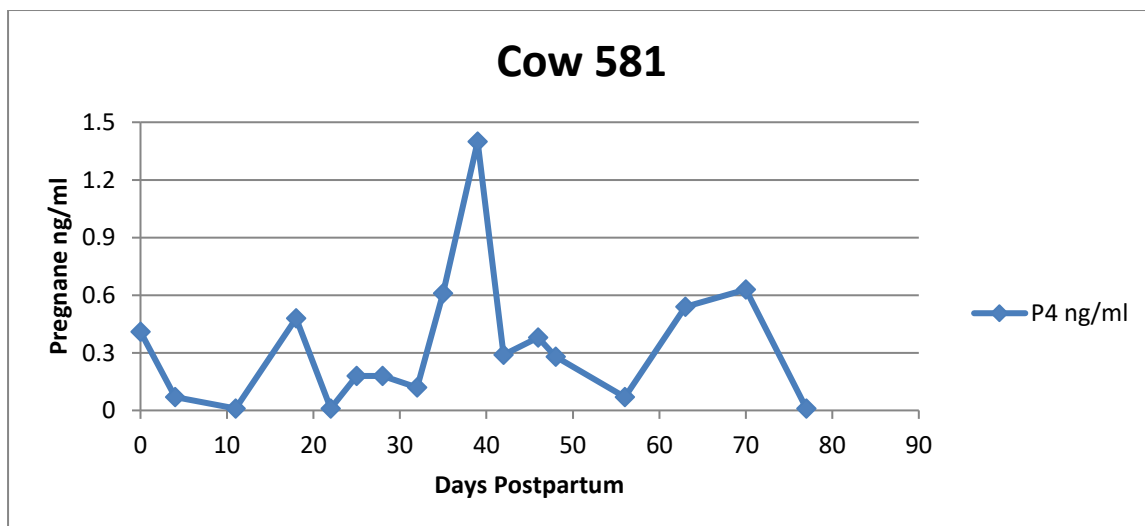
DOV2

- First rise of pregnane (>0.3ng/ml) occurred at day 29 postpartum; 0.38ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(53 - 32) + 3.5 = 24.5$ days
- CLA : $(T1 - 1.75)$; $(29 - 1.75) = 27.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



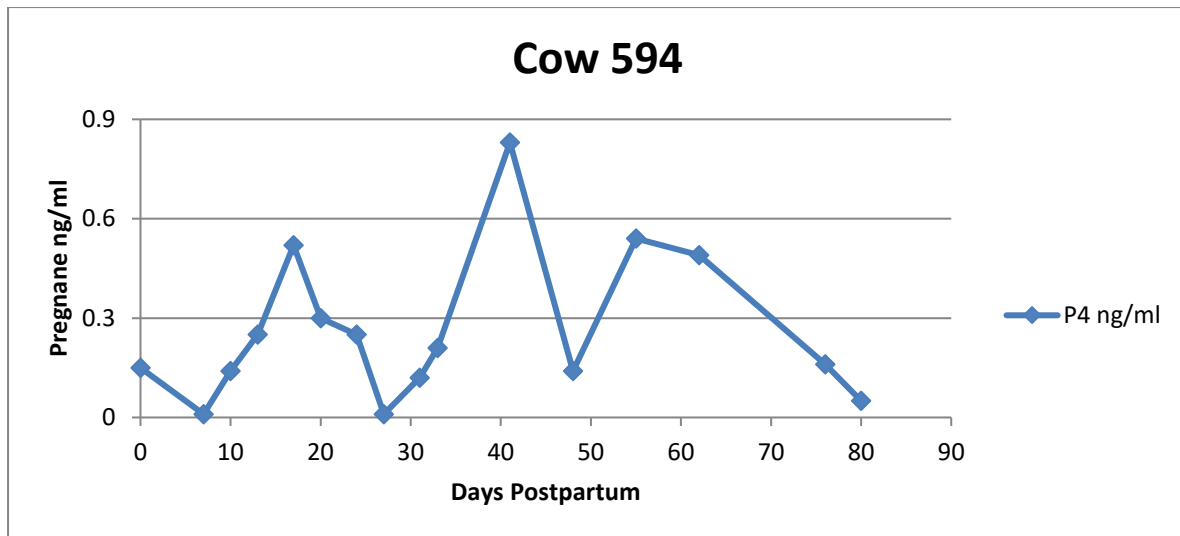
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 36; 0.70ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(43 - 36) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(54 - 47) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(36 - 1.75) = 34.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(64 - 56) + 3.5 = 11.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



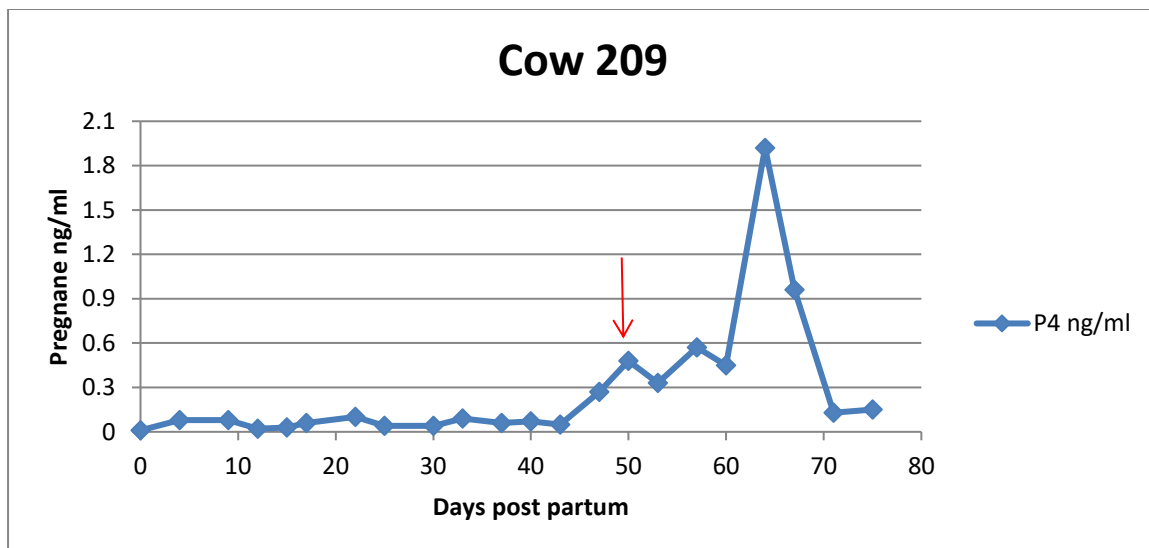
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.48ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(32 - 22) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(39 - 35) + 3.5 = 7.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



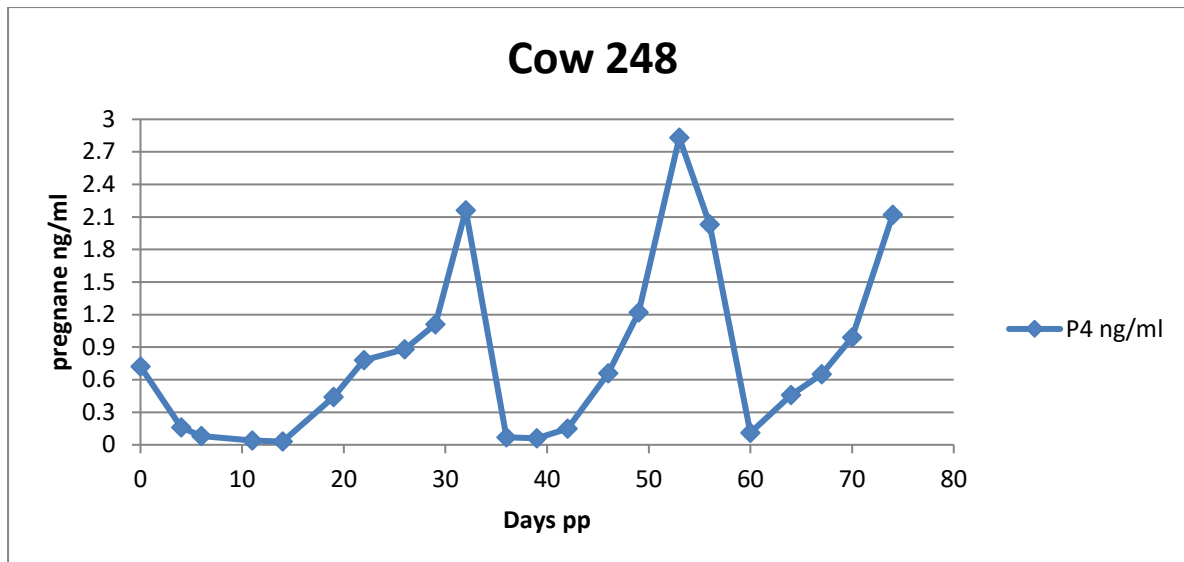
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 17; 0.52ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(20 - 17) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(33 - 24) + 3.5 = 12.5$ days
- CLA : $(T1 - 1.75)$; $(17 - 1.75) = 15.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



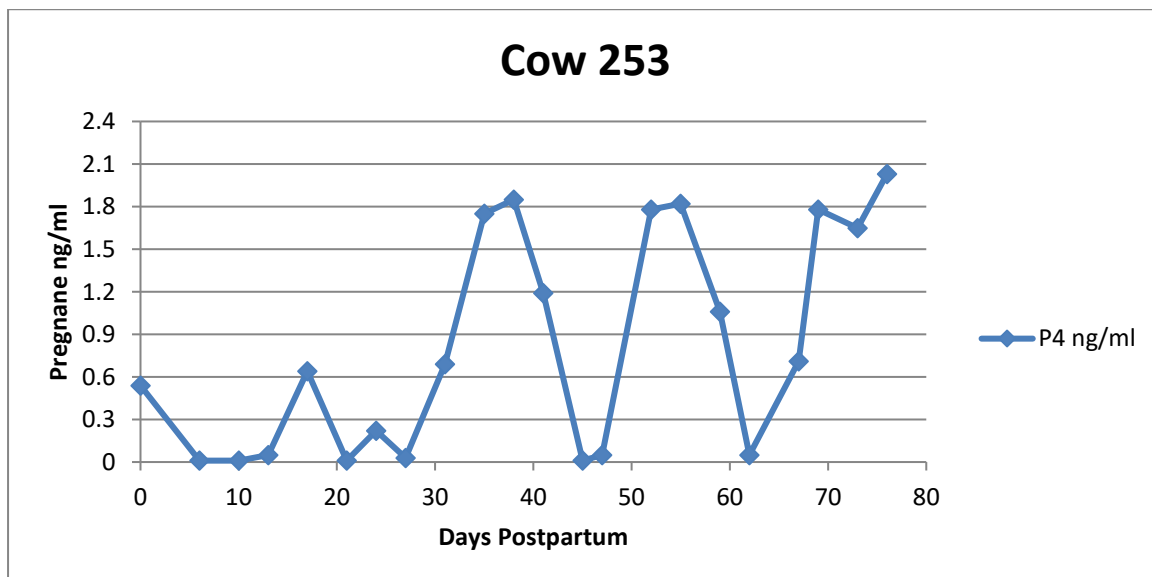
PCL1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 50 postpartum; 0.48ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(67 - 50) + 3.5 = 20.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(75 - 71) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(50 - 1.75) = 48.25$ days



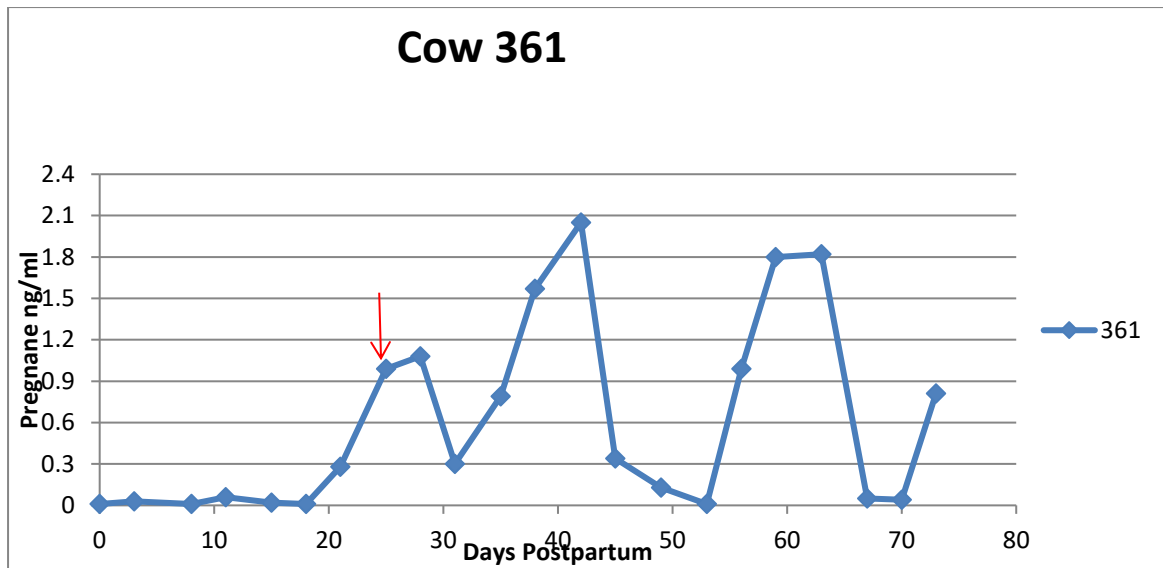
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 19; 0.44ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(32 - 19) + 3.5 = 16.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(42 - 36) + 3.5 = 9.5$ days
- CLA : $(T1 - 1.75)$; $(19 - 1.75) = 17.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(56 - 46) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



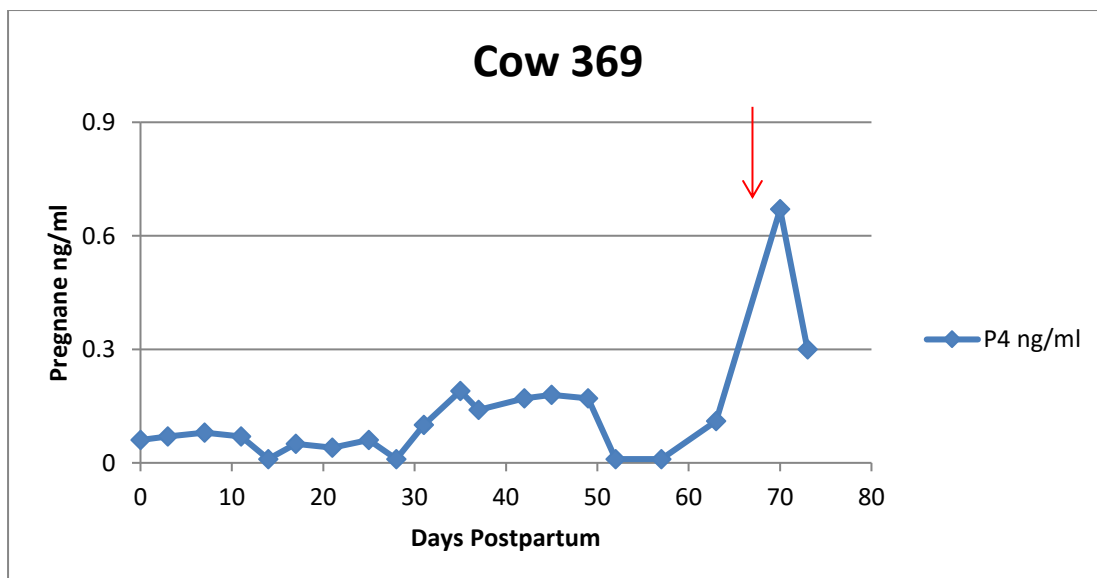
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 17; 0.64ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(27 - 21) + 3.5 = 9.5$ days
- CLA : $(T1 - 1.75)$; $(17 - 1.75) = 15.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(41 - 31) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(47 - 45) + 3.5 = 5.5$ days



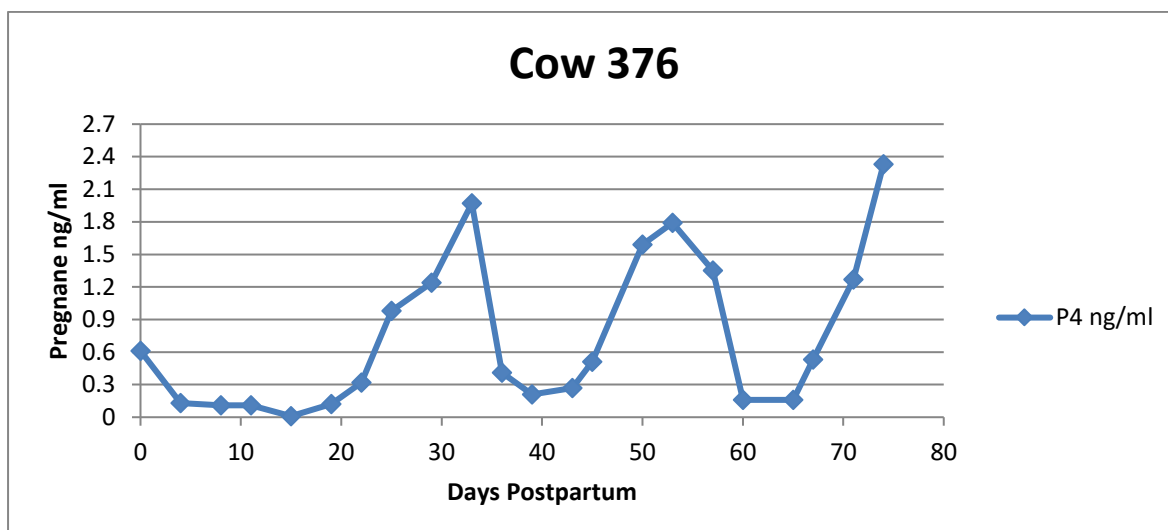
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 25; 0.99ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(45 - 25) + 3.5 = 23.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(53 - 49) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(25 - 1.75) = 23.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(63 - 56) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(70 - 67) + 3.5 = 6.5$ days



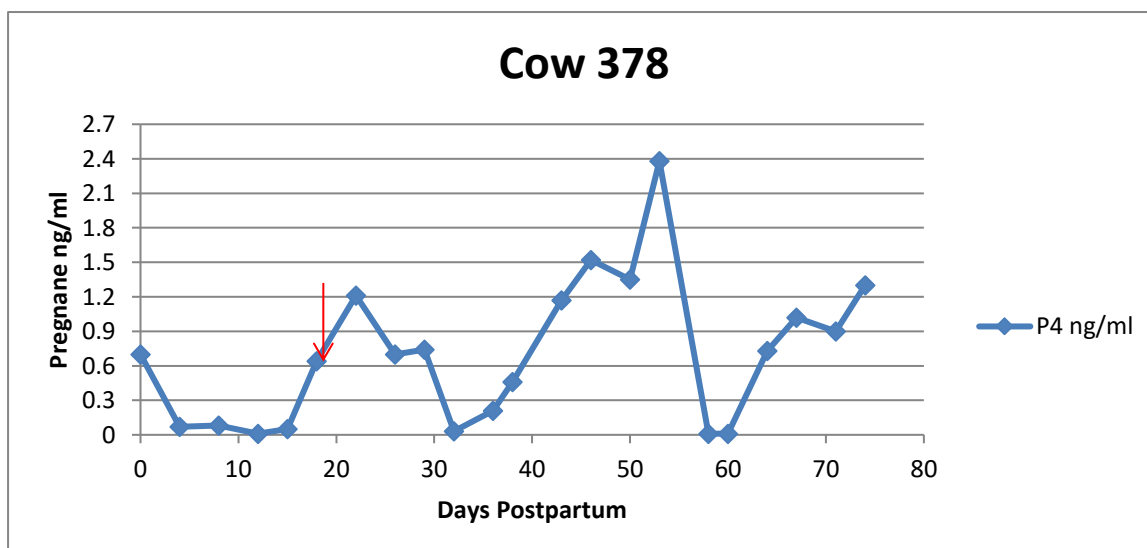
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 70 postpartum; 0.67ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$;
- 1st ILI : $(T6 - T5 + 3.5)$;
- CLA : $(T1 - 1.75)$; $(70 - 1.75) = 68.25$ days



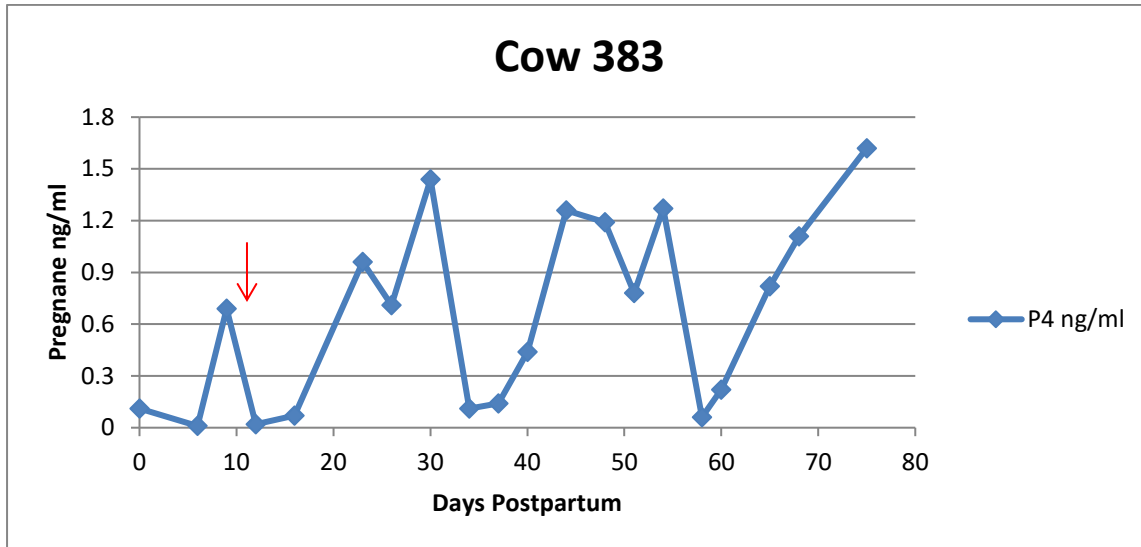
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 22; 0.32ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(36 - 22) + 3.5 = 17.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(43 - 39) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(22 - 1.75) = 20.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(57 - 45) + 3.5 = 15.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(65 - 60) + 3.5 = 8.5$ days



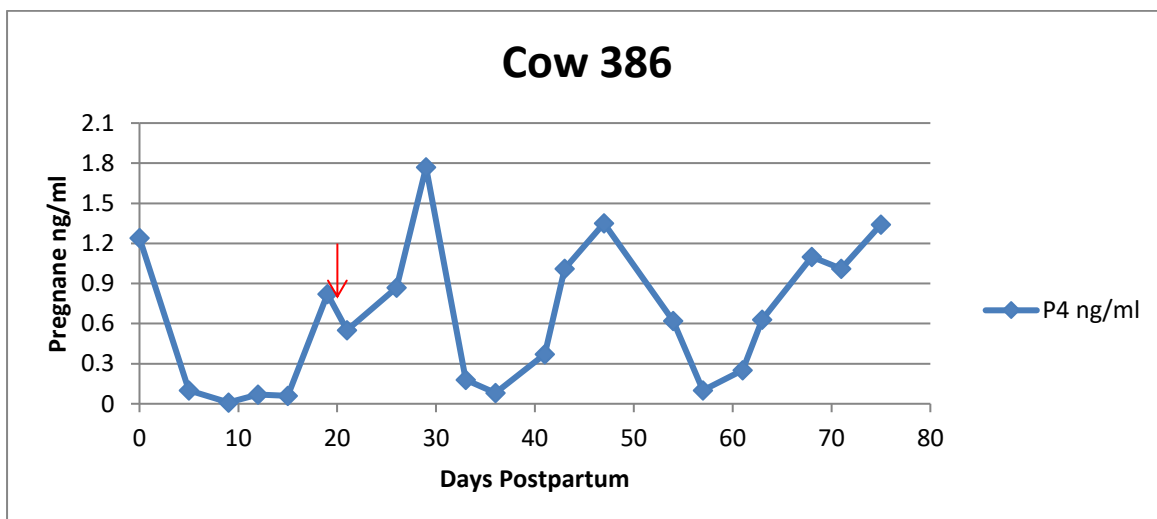
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.64ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 18) + 3.5 = 14.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(36 - 32) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(53 - 38) + 3.5 = 18.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(60 - 58) + 3.5 = 5.5$ days



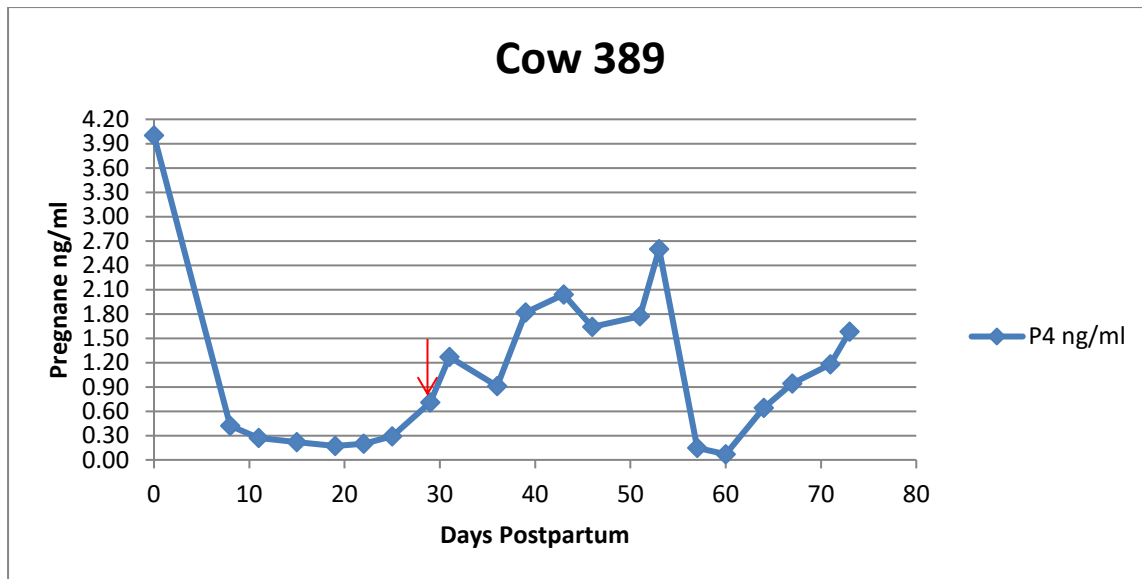
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 9; 0.69ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(16 - 12) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(9 - 1.75) = 7.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(30 - 23) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(37 - 34) + 3.5 = 6.5$ days



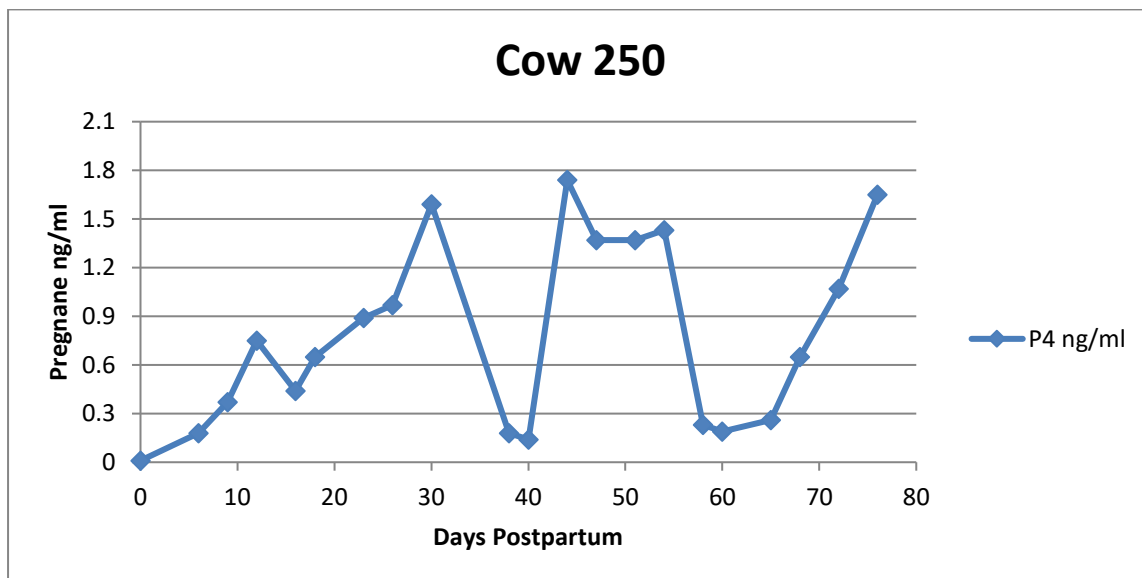
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 19; 0.82ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 19) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(36 - 33) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(19 - 1.75) = 17.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(54 - 41) + 3.5 = 16.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(61 - 57) + 3.5 = 7.5$ days



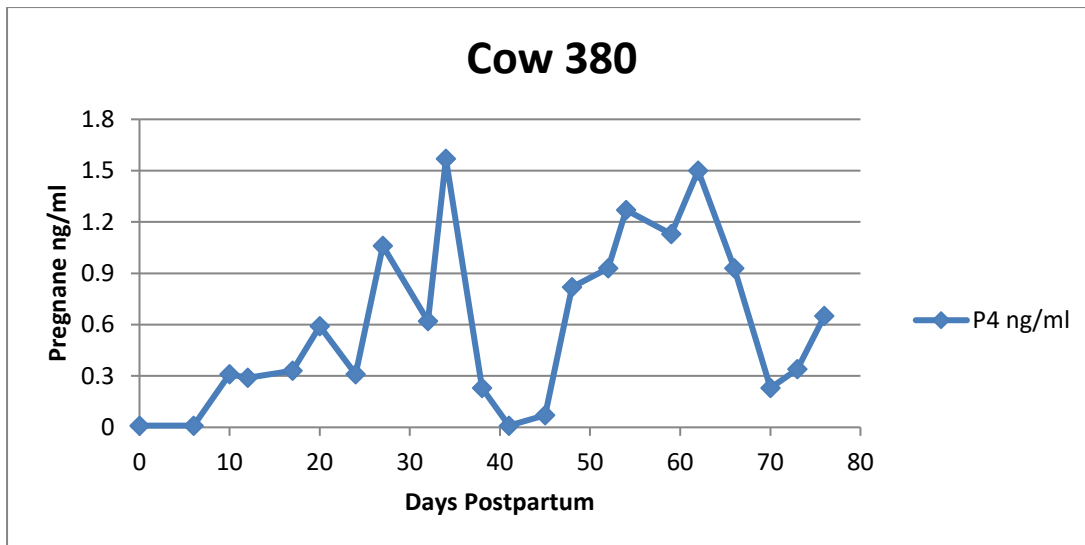
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 29; 0.71ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(53 - 29) + 3.5 = 27.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(60 - 57) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(29 - 1.75) = 27.25$ days



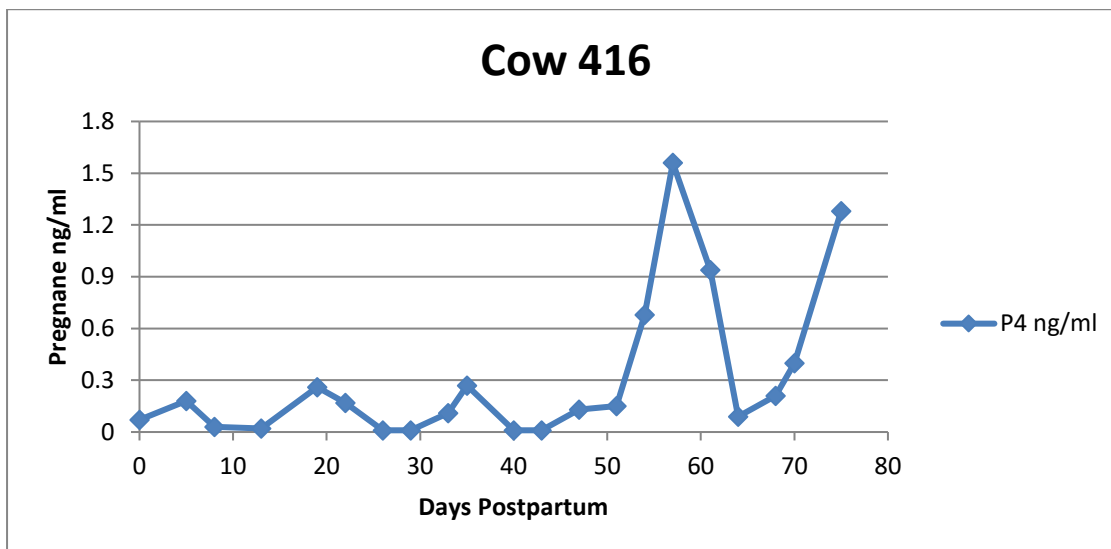
PCL1

- First rise of pregnane (>0.3ng/ml) occurred at day 9 postpartum; 0.37ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(30 - 9) + 3.5 = 24.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(40 - 38) + 3.5 = 5.5$ days
- CLA : $(T1 - 1.75)$; $(9 - 1.75) = 7.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(54 - 44) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(65 - 58) + 3.5 = 10.5$ days



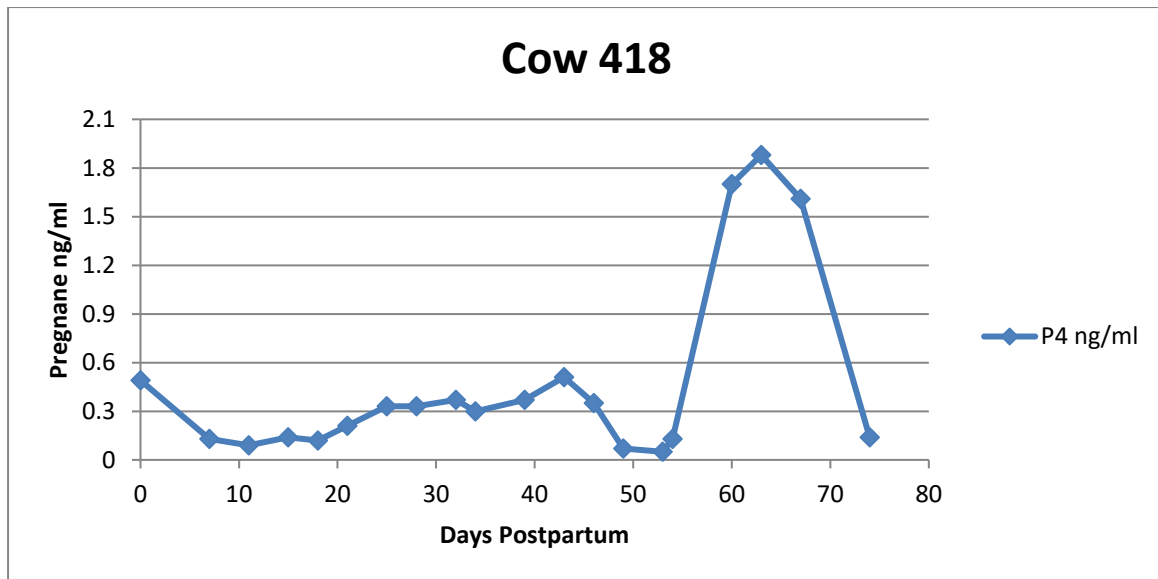
PCL1

- First rise of pregnane (>0.3ng/ml) occurred at day 10 postpartum; 0.31ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(10 - 1.75) = 8.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(34 - 17) + 3.5 = 20.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(45 - 38) + 3.5 = 10.5$ days



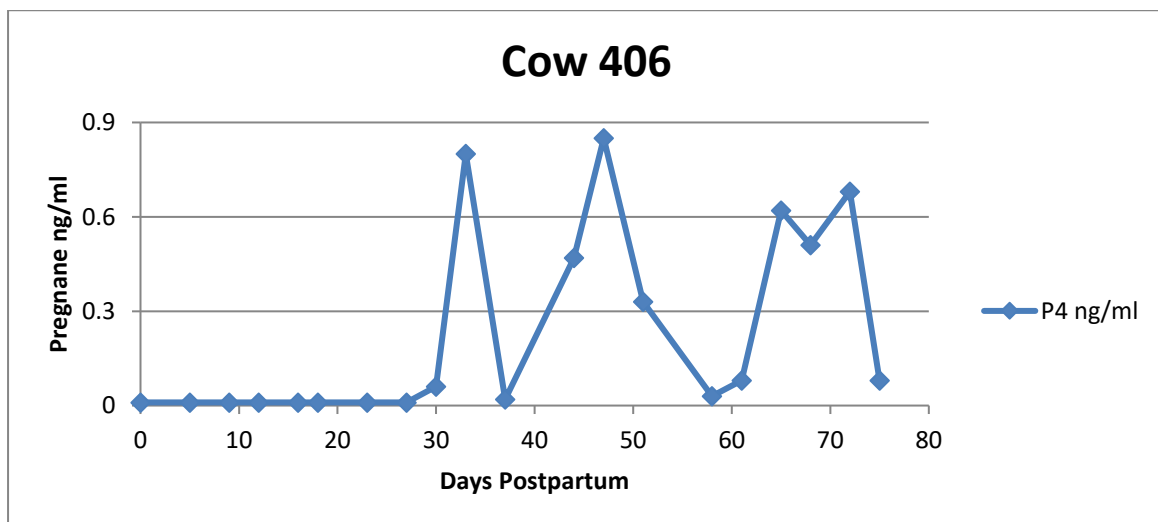
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 54 postpartum; 0.68ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(61 - 54) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(68 - 64) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(54 - 1.75) = 52.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



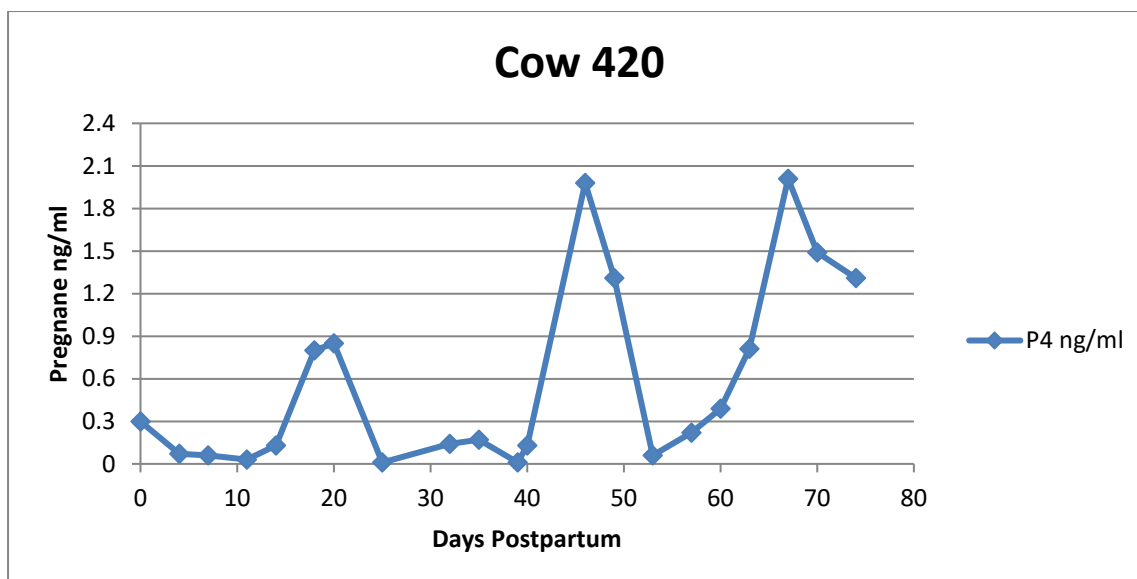
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 25; 0.33ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(46 - 25) + 3.5 = 24.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(54 - 49) + 3.5 = 8.5$ days
- CLA : $(T1 - 1.75)$; $(25 - 1.75) = 23.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(67 - 60) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



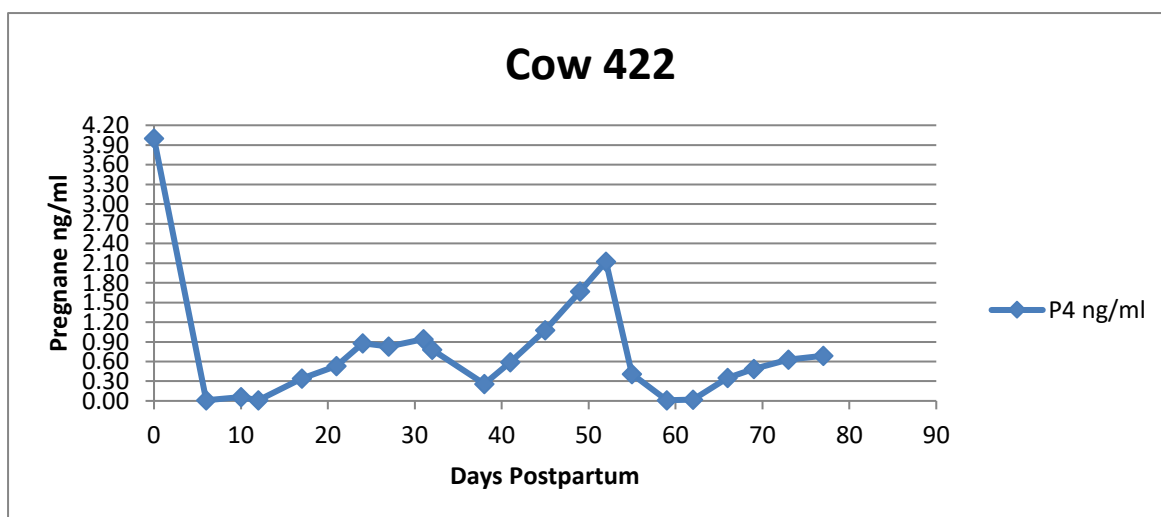
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 33; 1.39ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(33 - 1.75) = 31.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(51 - 44) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(61 - 58) + 3.5 = 6.5$ days



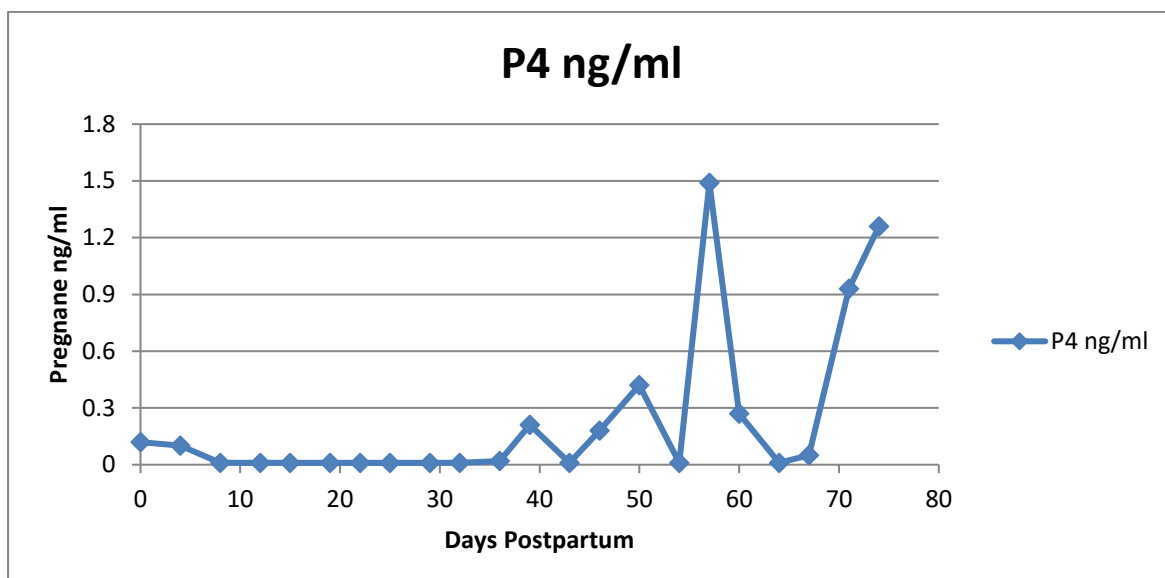
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.80ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(20 - 18) + 3.5 = 5.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(40 - 25) + 3.5 = 18.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(49 - 46) + 3.5 = 6.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(57 - 53) + 3.5 = 7.5$ days



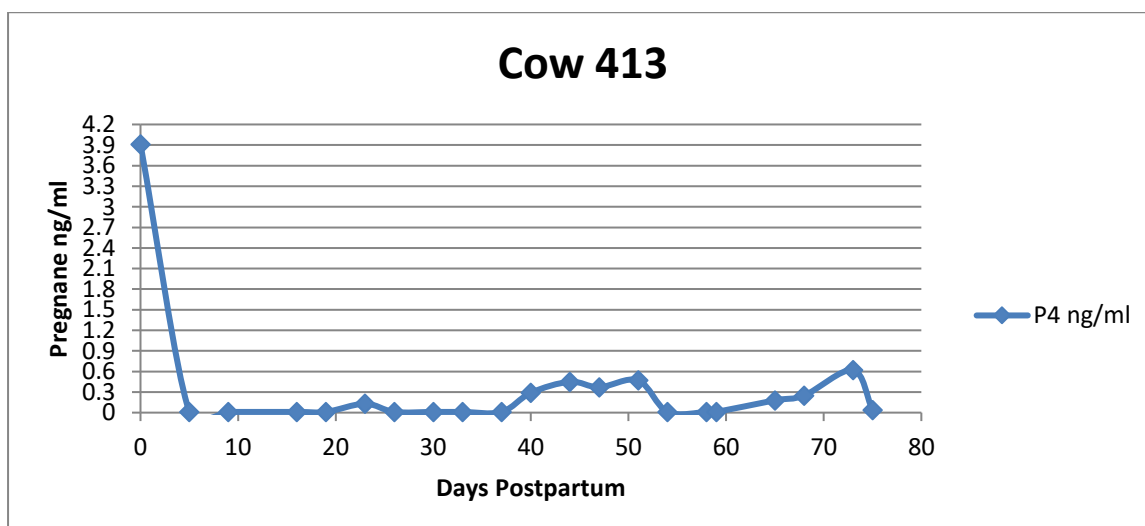
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 17; 0.34ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(32 - 17) + 3.5 = 18.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(17 - 1.75) = 15.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(55 - 41) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(62 - 59) + 3.5 = 6.5$ days



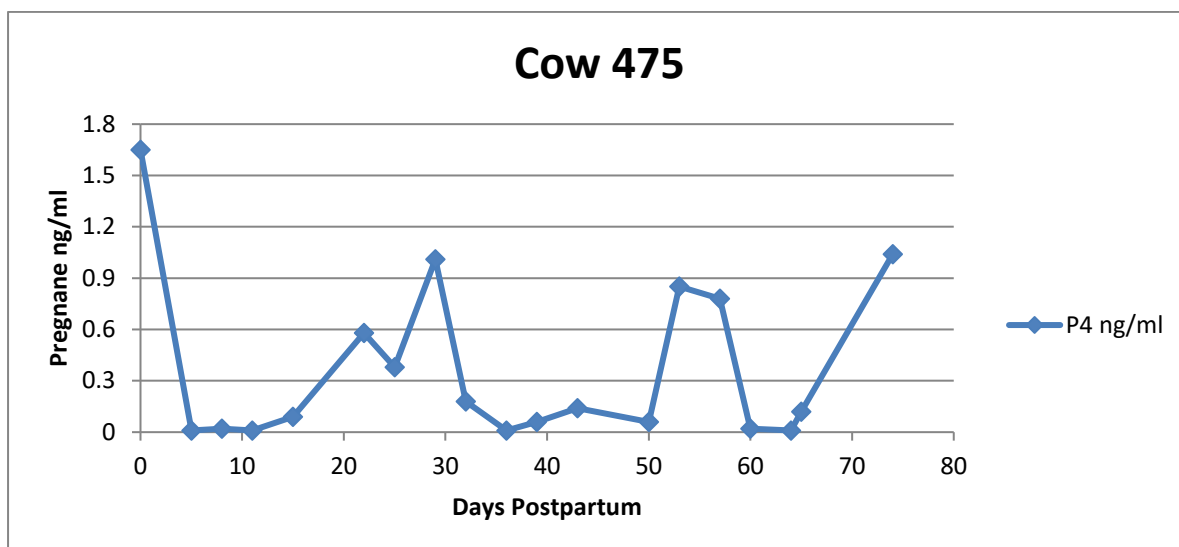
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 50 postpartum; 0.42ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(50 - 1.75) = 48.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(67 - 60) + 3.5 = 10.5$ days



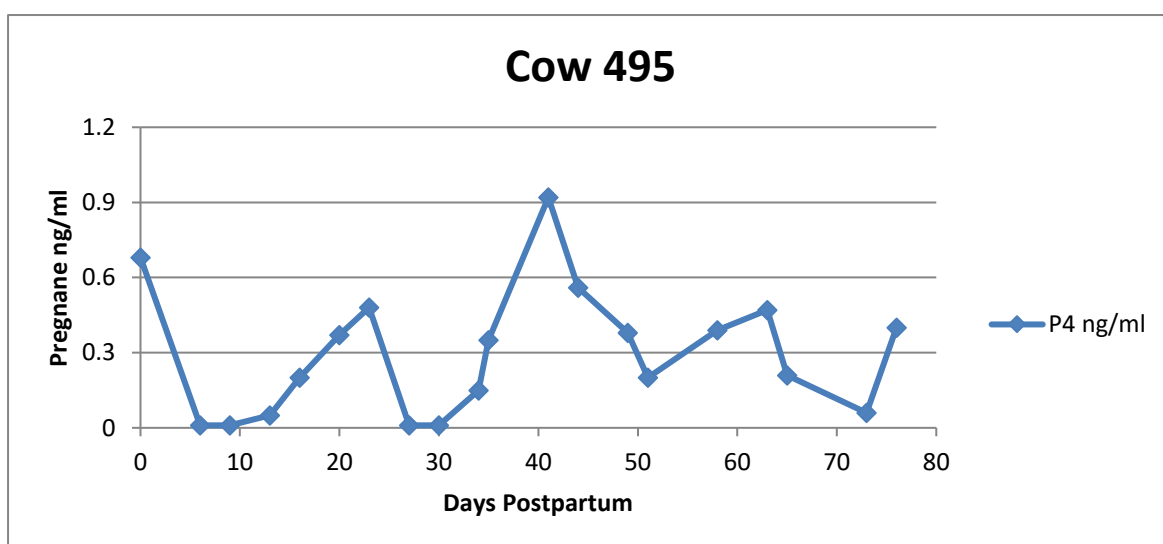
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 44; 0.45ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(51 - 44) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(68 - 54) + 3.5 = 17.5$ days
- CLA : $(T1 - 1.75)$; $(44 - 1.75) = 42.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



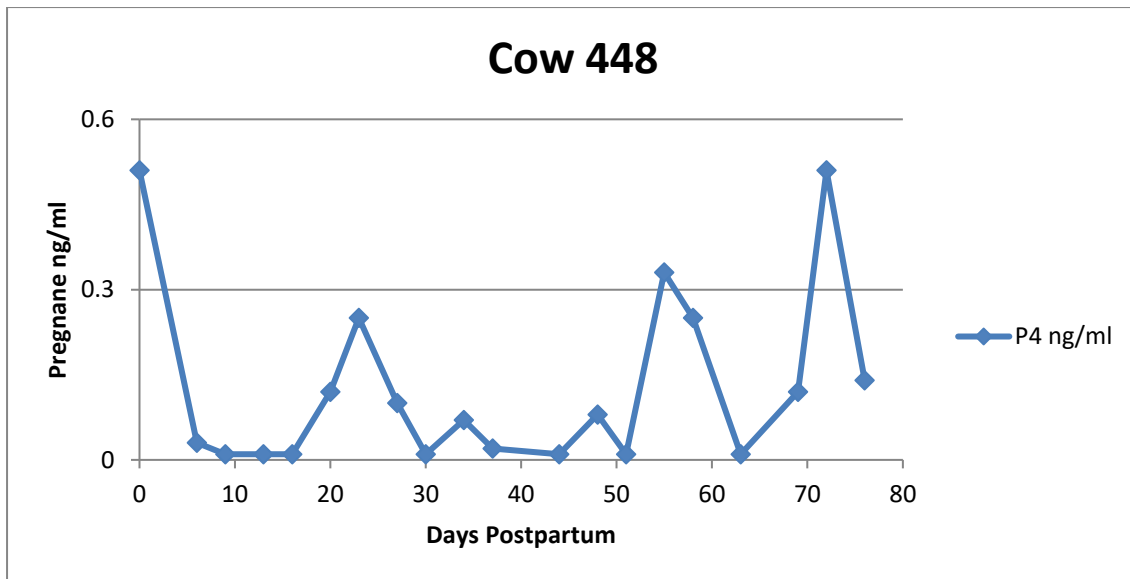
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 22; 0.58ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 22) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(50 - 32) + 3.5 = 21.5$ days
- CLA : $(T1 - 1.75)$; $(22 - 1.75) = 20.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(57 - 53) + 3.5 = 7.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(65 - 60) + 3.5 = 8.5$ days



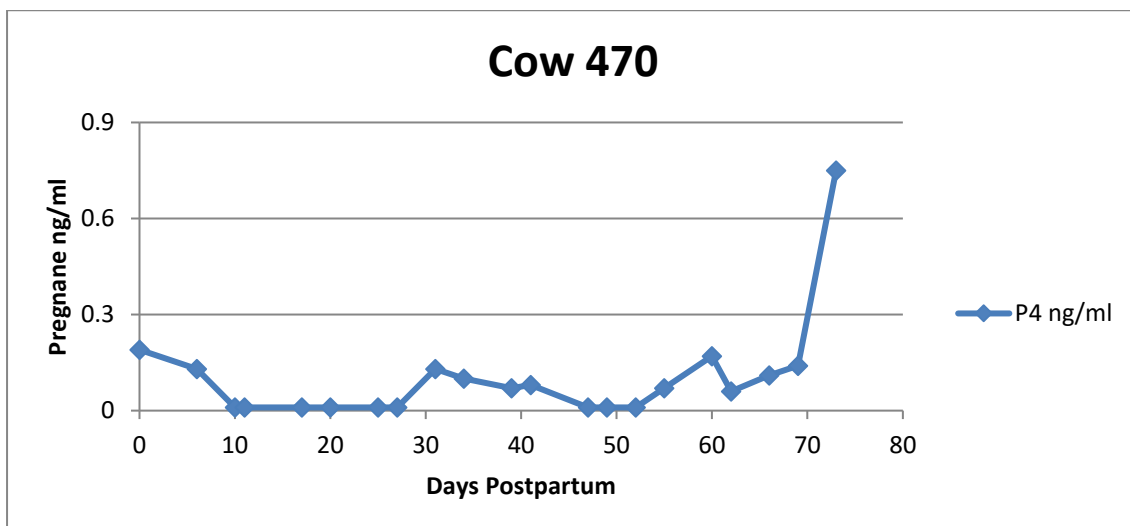
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 20; 0.37ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(23 - 20) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(34 - 27) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(20 - 1.75) = 18.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(49 - 35) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $= 3.5$ days



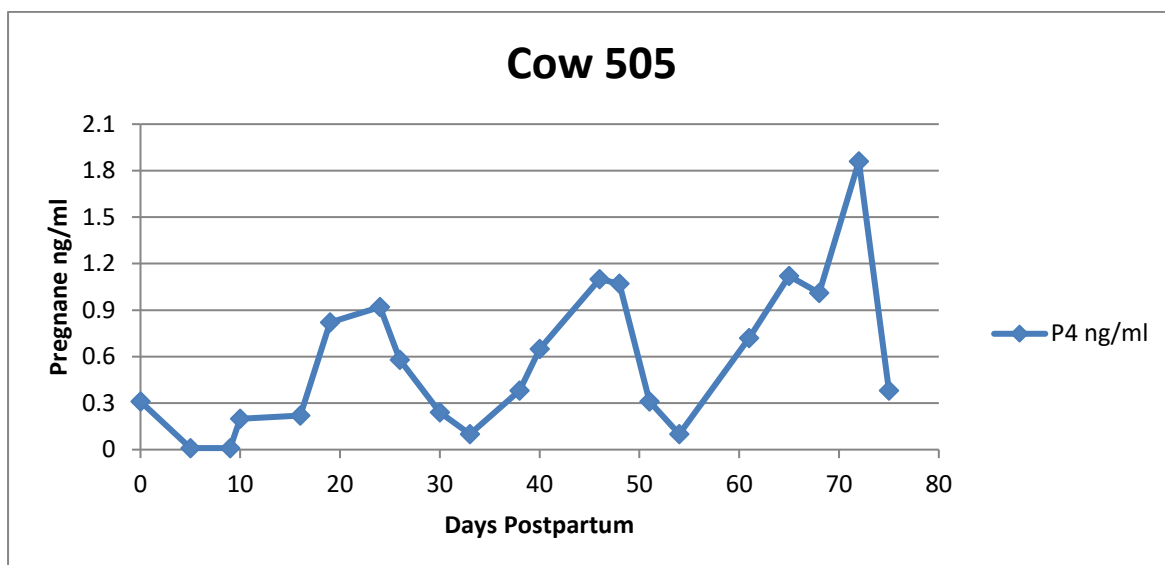
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 55 postpartum; 0.33ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(69 - 58) + 3.5 = 14.5$ days
- CLA : $(T1 - 1.75)$; $(55 - 1.75) = 53.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$



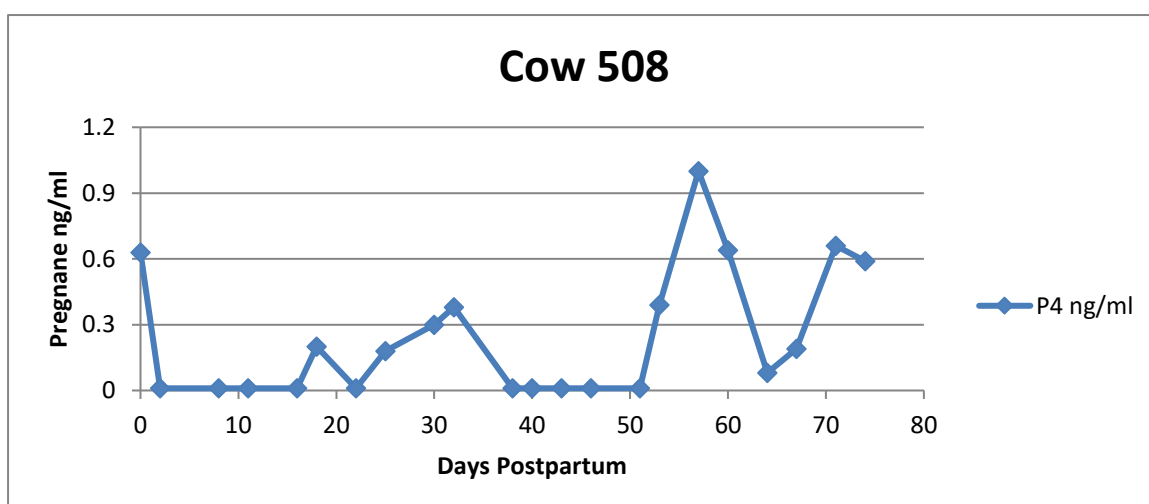
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 73 postpartum; 0.75ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$
- CLA : $(T1 - 1.75)$; $(73 - 1.75) = 71.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



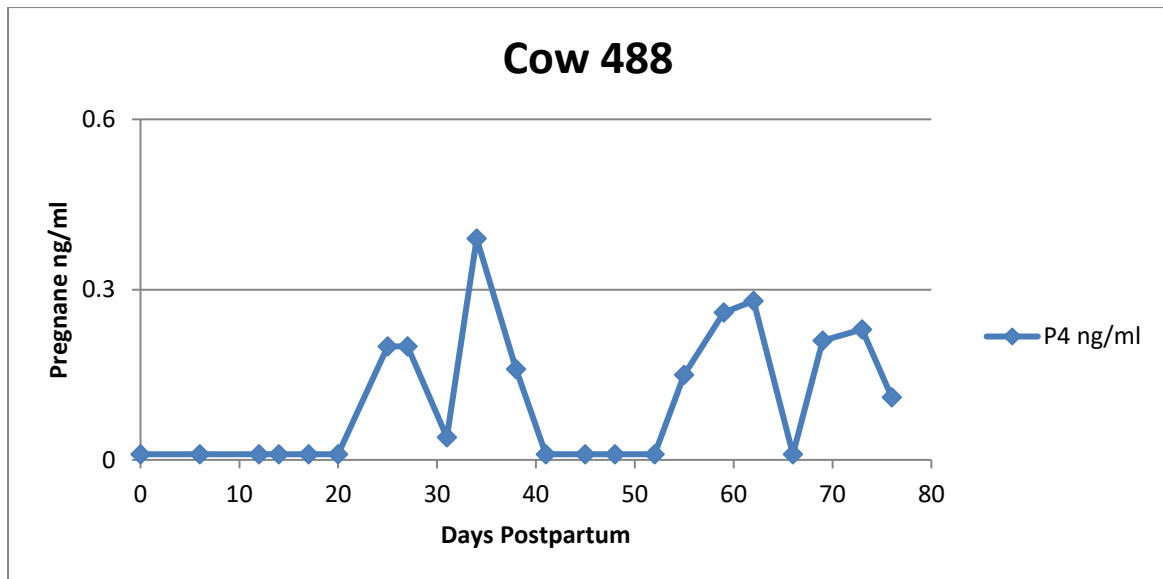
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 19; 0.82ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(26 - 19) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(33 - 30) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(19 - 1.75) = 17.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(51 - 38) + 3.5 = 16.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



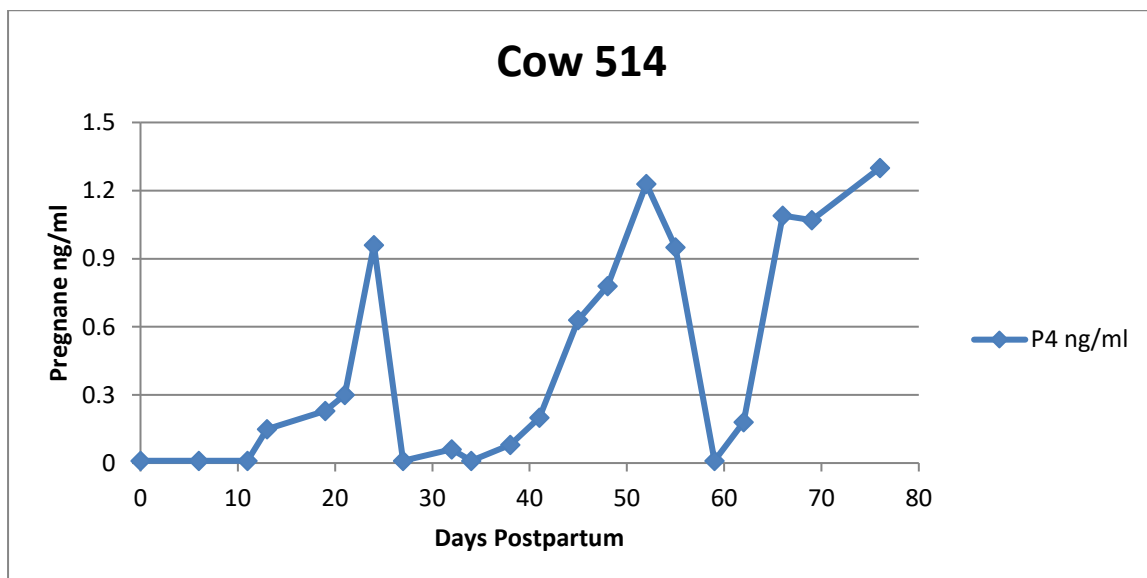
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 30; 0.30ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(32 - 30) + 3.5 = 5.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(51 - 38) + 3.5 = 16.5$ days
- CLA : $(T1 - 1.75)$; $(30 - 1.75) = 28.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(60 - 53) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(67 - 64) + 3.5 = 6.5$ days



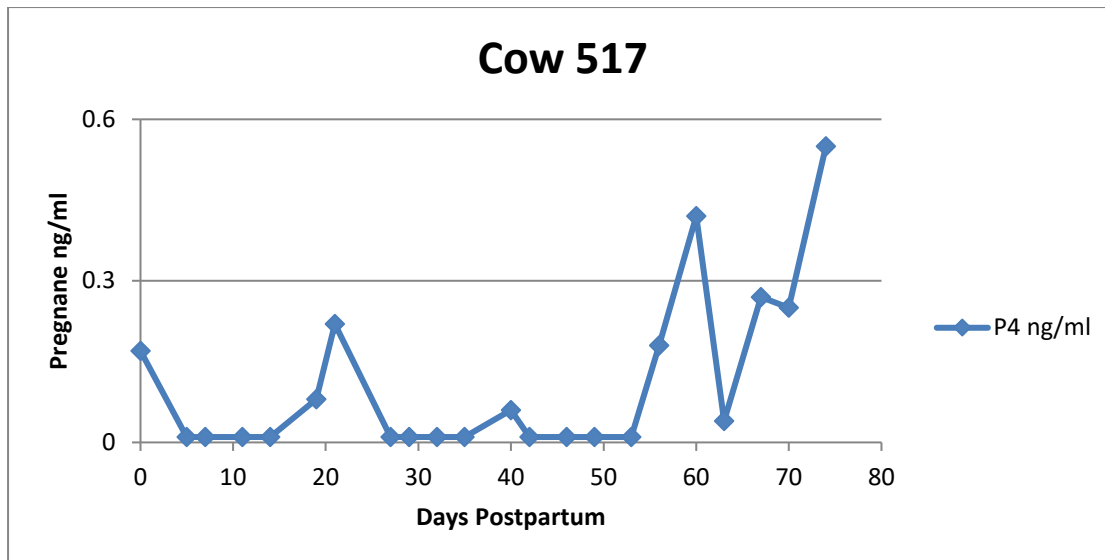
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 34 postpartum; 0.39ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(76 - 38) + 3.5 = 41.5$ days
- CLA : $(T1 - 1.75)$; $(34 - 1.75) = 32.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



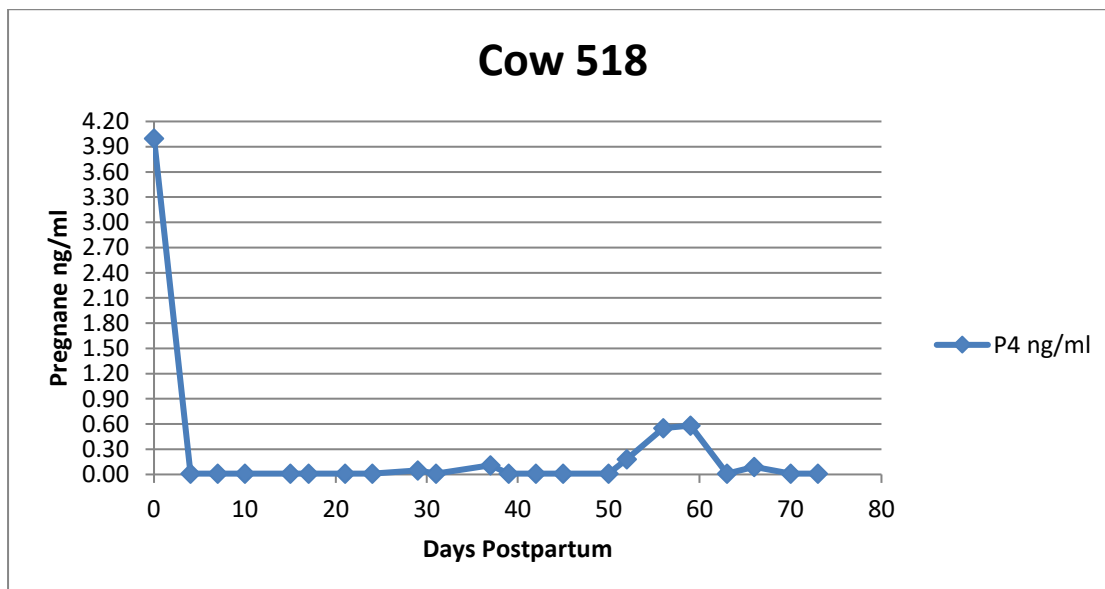
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 21; 0.30ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(24 - 21) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(41 - 27) + 3.5 = 17.5$ days
- CLA : $(T1 - 1.75)$; $(21 - 1.75) = 19.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(55 - 45) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(62 - 59) + 3.5 = 6.5$ days



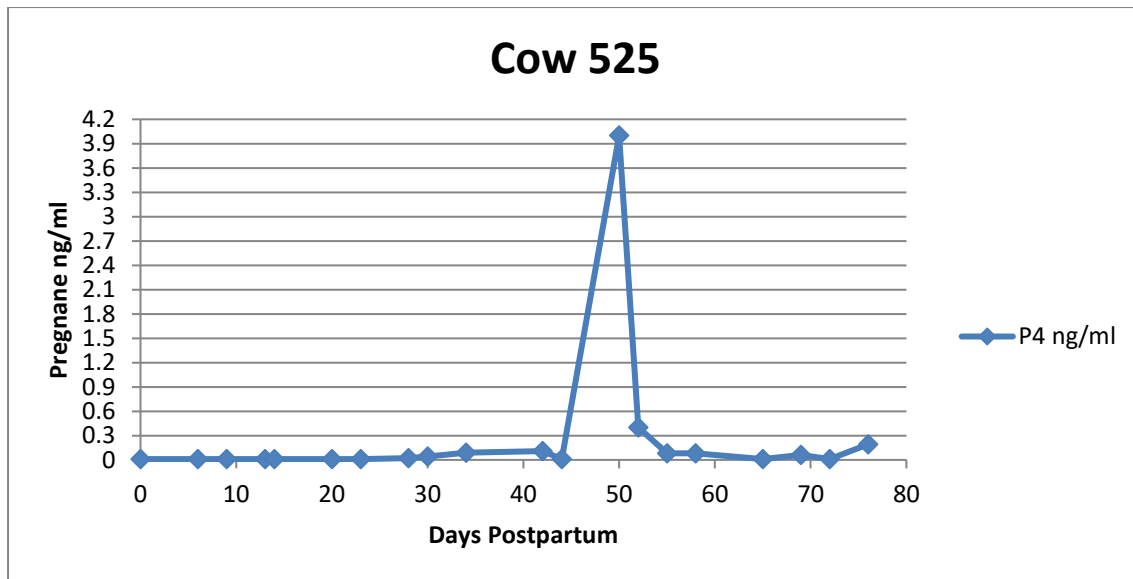
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 60 postpartum; 0.42ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(70 - 63) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(60 - 1.75) = 58.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



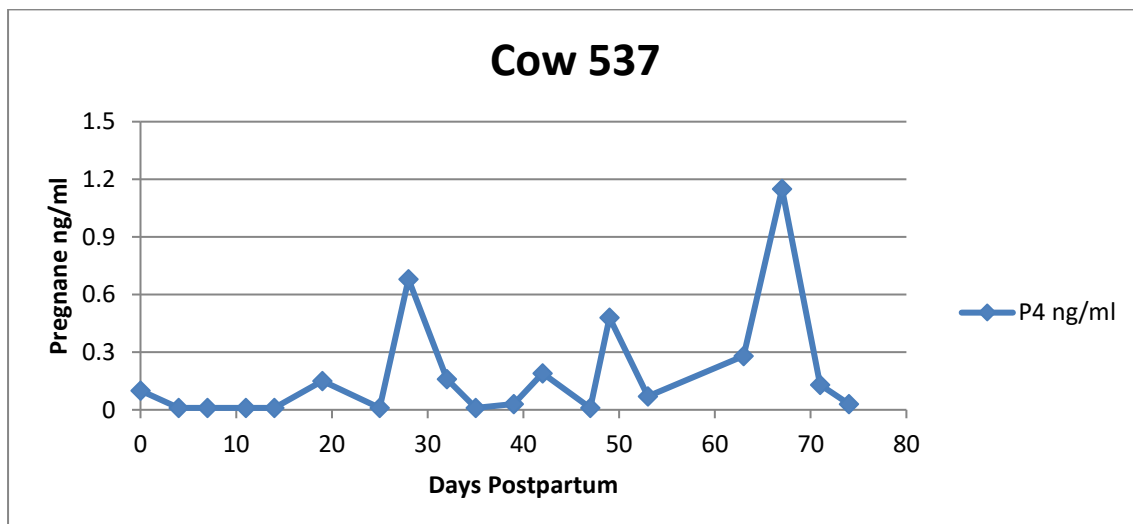
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 56 postpartum; 0.55ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(59 - 56) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(73 - 63) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(56 - 1.75) = 54.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



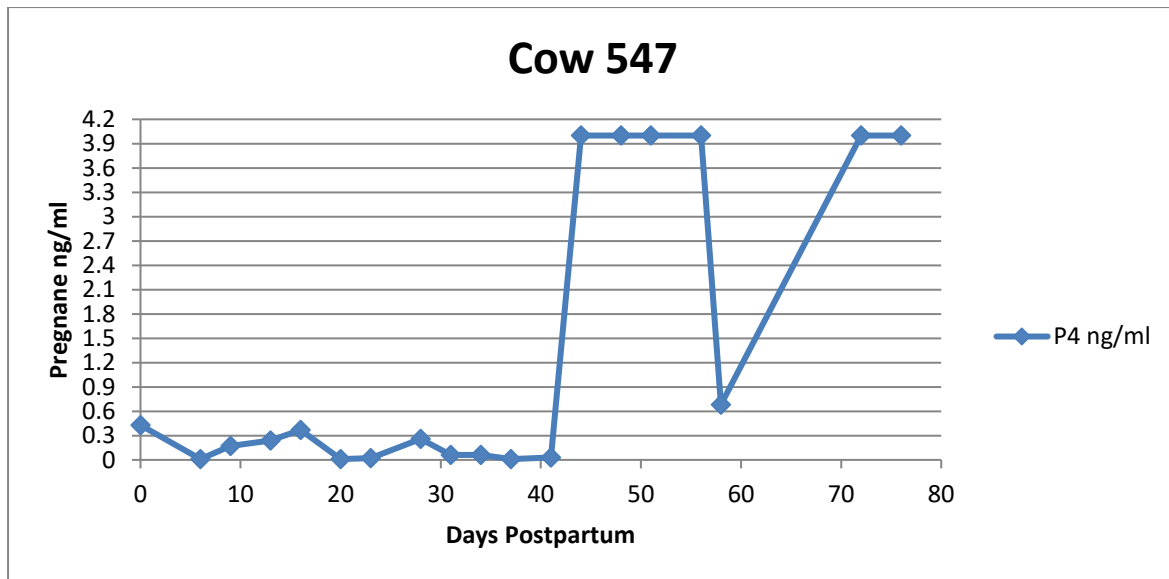
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 50 postpartum; 4.00ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(52 - 50) + 3.5 = 5.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(76 - 55) + 3.5 = 24.5$ days
- CLA : $(T1 - 1.75)$; $(50 - 1.75) = 48.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



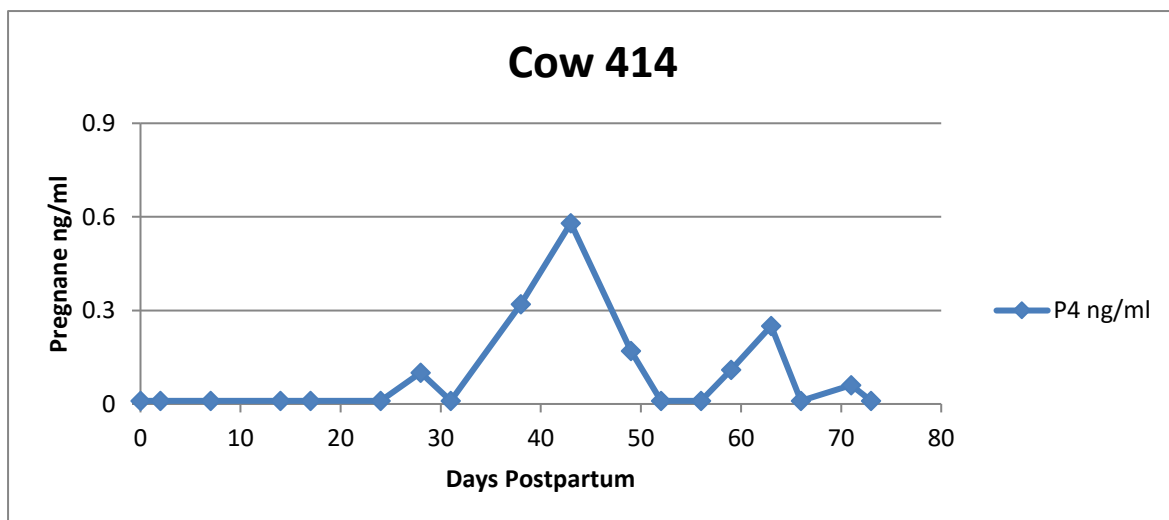
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 28; 0.68ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(47 - 32) + 3.5 = 18.5$ days
- CLA : $(T1 - 1.75)$; $(28 - 1.75) = 26.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(63 - 53) + 3.5 = 13.5$ days



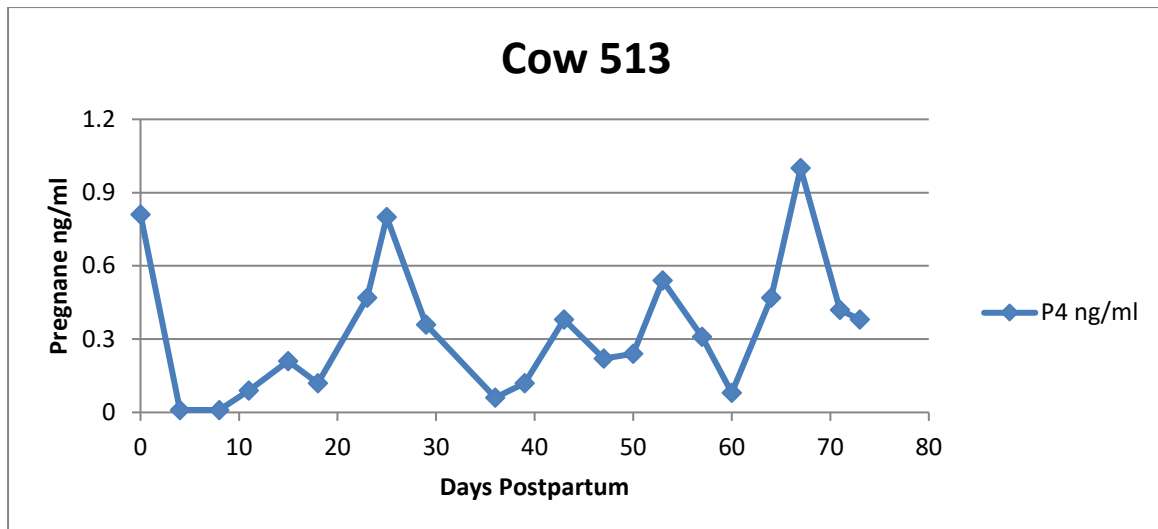
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 16; 0.37ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(41 - 20) + 3.5 = 24.5$ days
- CLA : $(T1 - 1.75)$; $(16 - 1.75) = 14.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



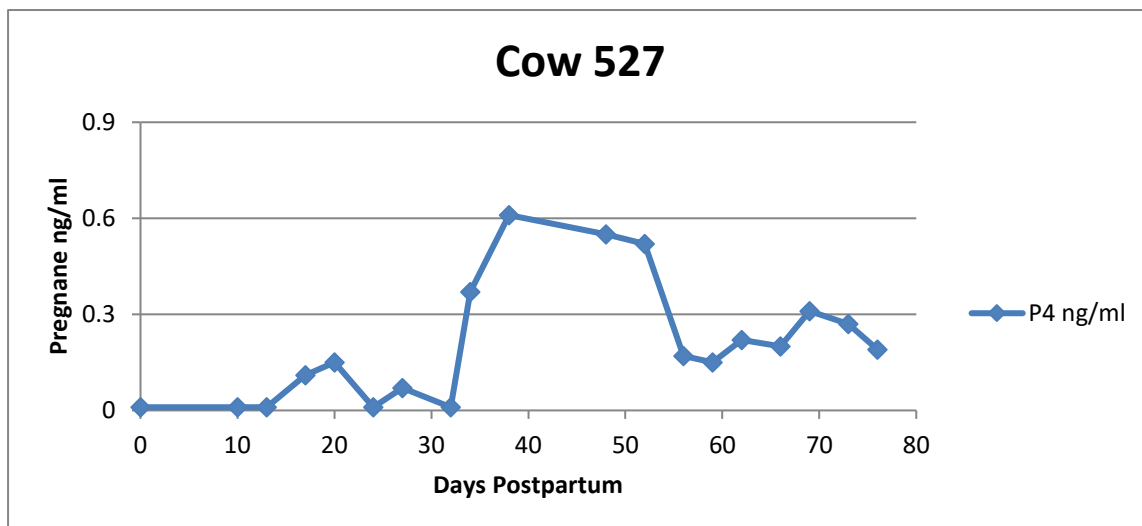
DOV2

- First rise of pregnane (>0.3ng/ml) occurred at day 38 postpartum; 0.32ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(43 - 38) + 3.5 = 8.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(73 - 49) + 3.5 = 27.5$ days
- CLA : $(T1 - 1.75)$; $(38 - 1.75) = 36.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



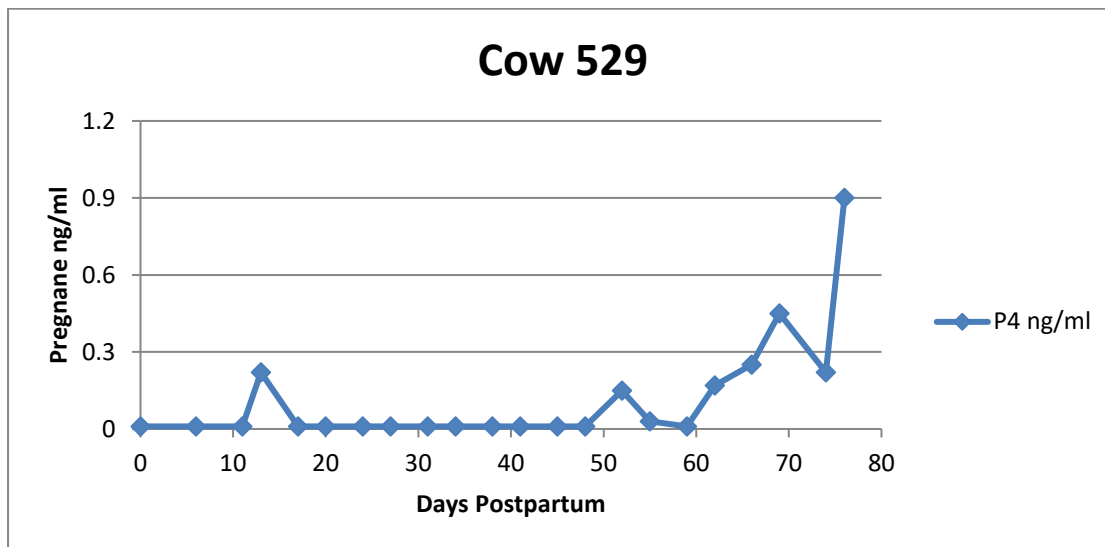
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 0.47ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 23) + 3.5 = 9.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(39 - 36) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(50 - 47) + 3.5 = 6.5$ days



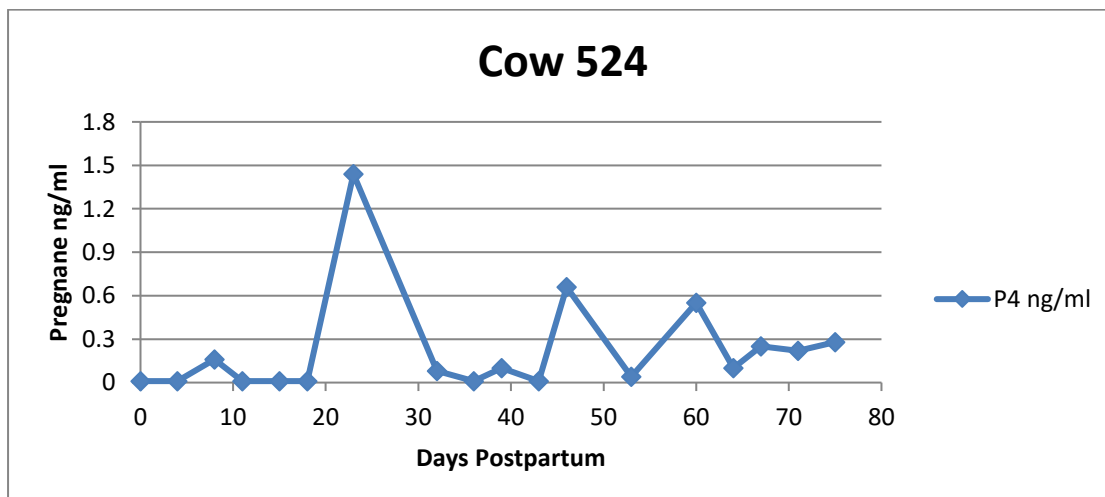
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 34; 0.37ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(52 - 34) + 3.5 = 21.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(66 - 56) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(34 - 1.75) = 32.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$



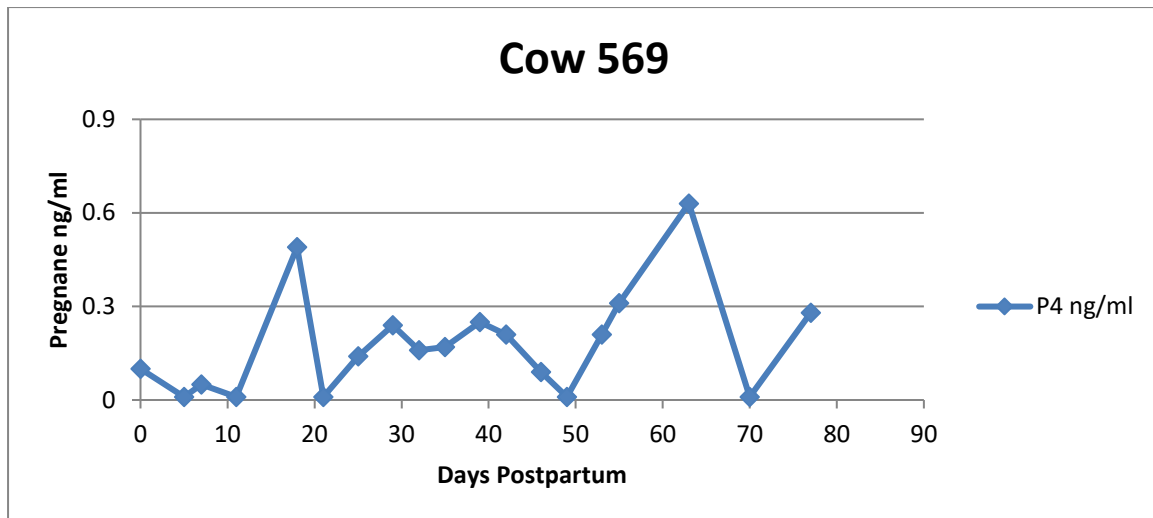
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 69 postpartum; 0.45ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(69 - 1.75) = 67.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



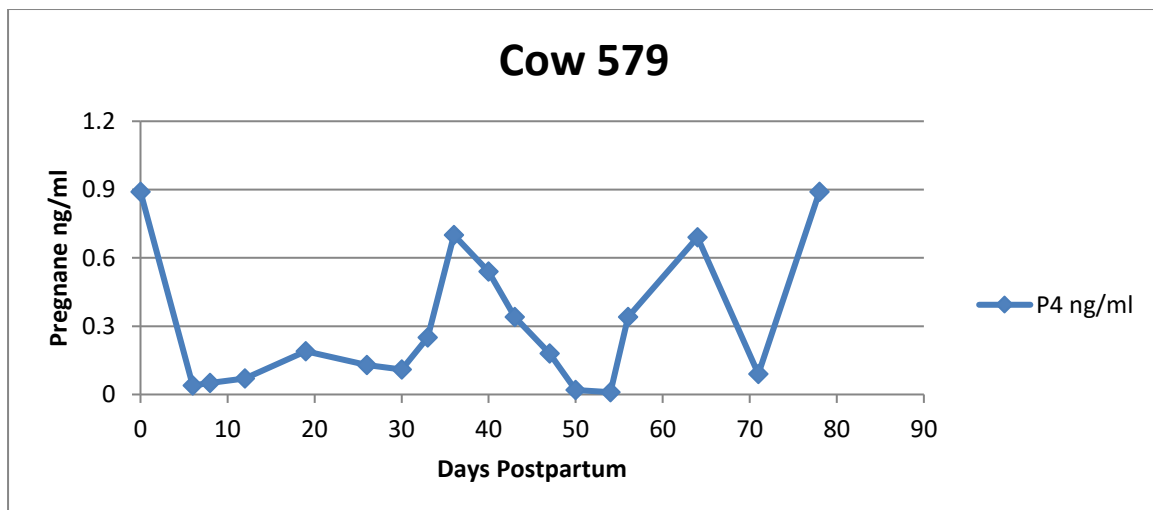
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 1.44ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(43 - 32) + 3.5 = 14.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



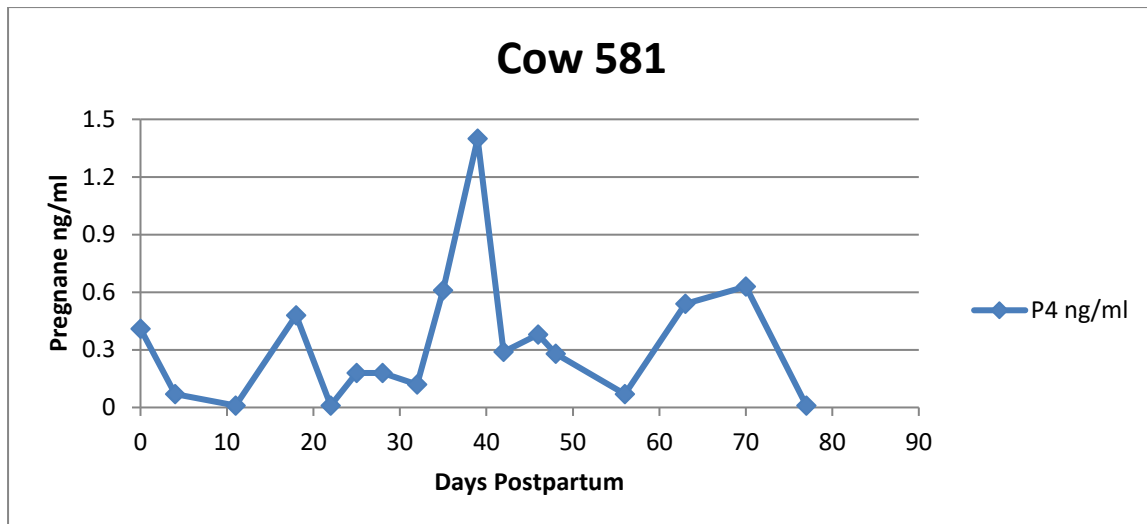
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.49ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(53 - 21) + 3.5 = 35.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(63 - 55) + 3.5 = 11.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



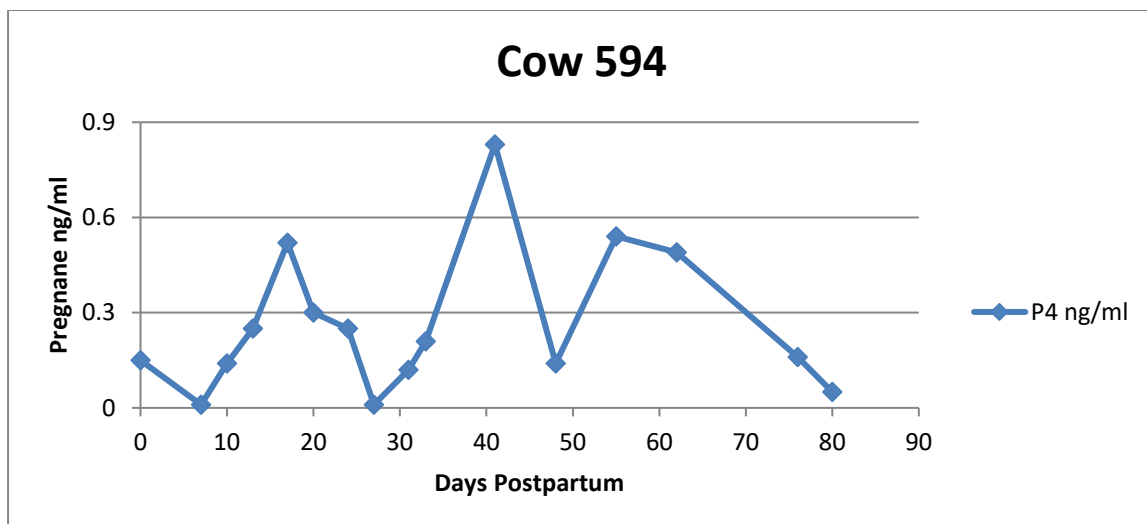
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 36; 0.70ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(43 - 36) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(54 - 47) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(36 - 1.75) = 34.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(64 - 56) + 3.5 = 11.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



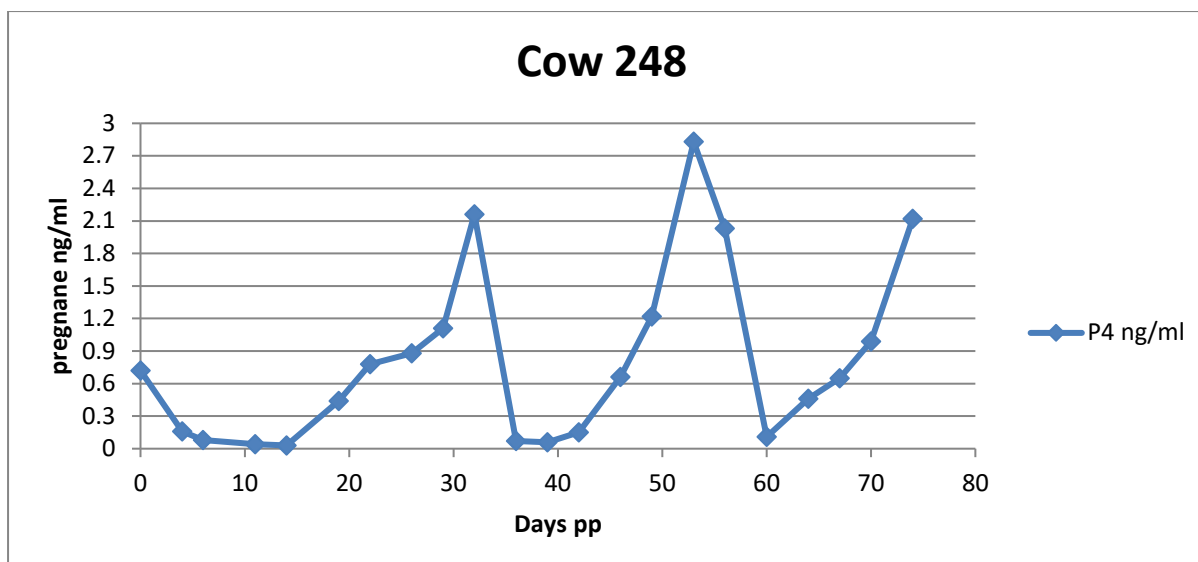
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.48ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(32 - 22) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(39 - 35) + 3.5 = 7.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



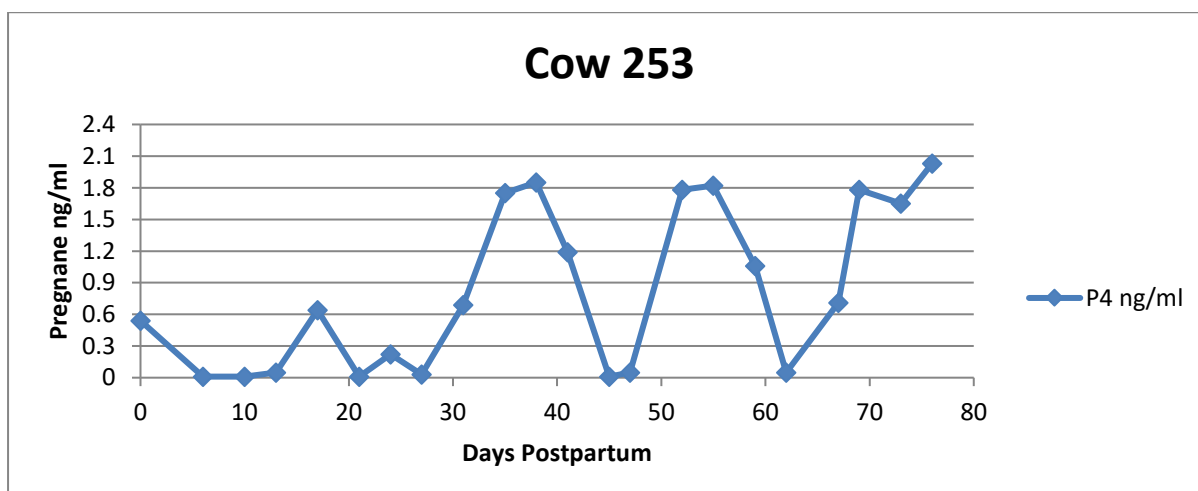
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 17; 0.52ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(20 - 17) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(33 - 24) + 3.5 = 12.5$ days
- CLA : $(T1 - 1.75)$; $(17 - 1.75) = 15.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



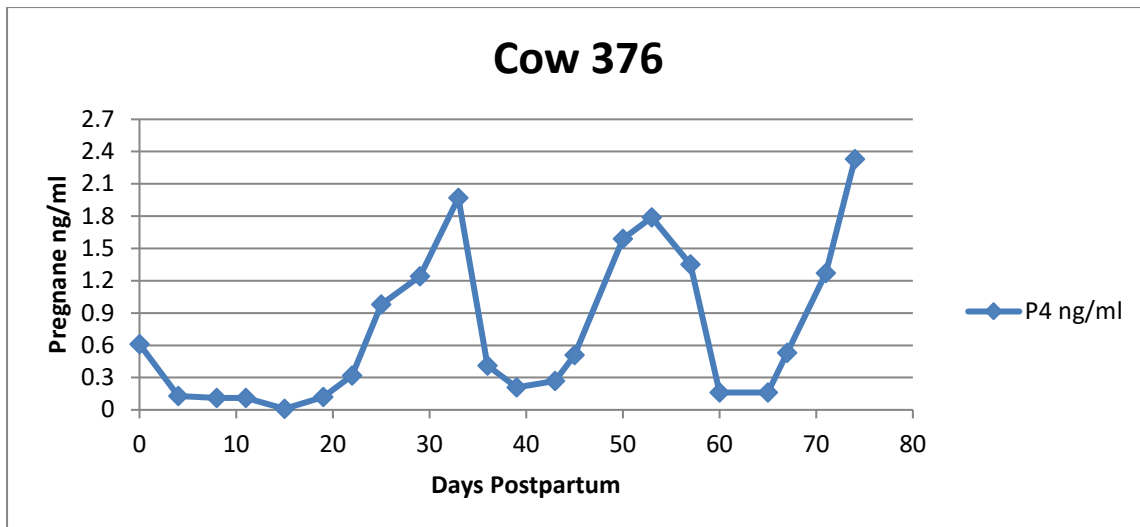
Normal

- First rise of pregnane ($>0.3\text{ng/ml}$) occurred within 45 days of postpartum (day 19; 0.44ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(32 - 19) + 3.5 = 16.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(42 - 36) + 3.5 = 9.5$ days
- CLA : $(T1 - 1.75)$; $(19 - 1.75) = 17.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(56 - 46) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



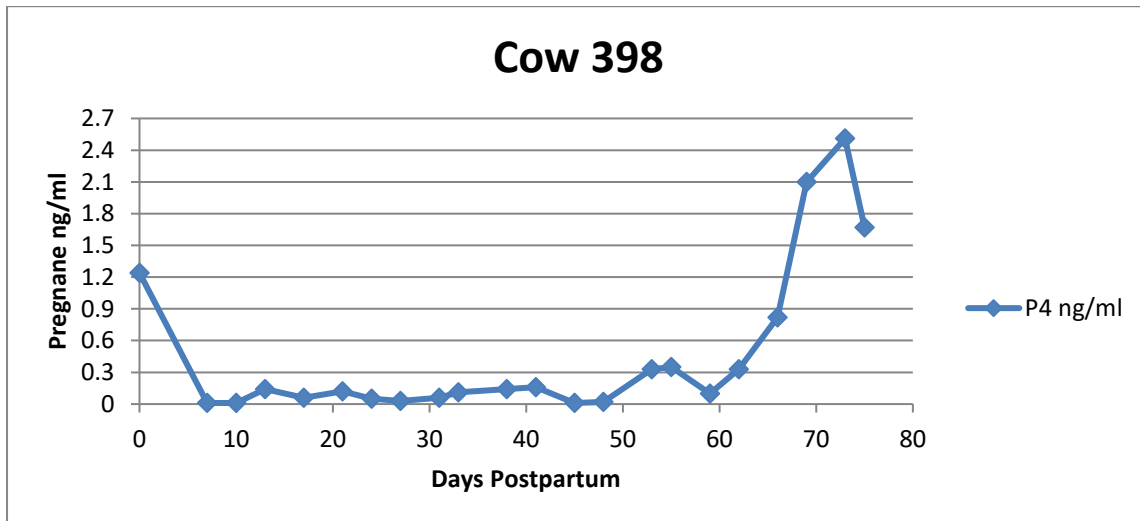
Normal

- First rise of pregnane ($>0.3\text{ng/ml}$) occurred within 45 days of postpartum (day 17; 0.64ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(27 - 21) + 3.5 = 9.5$ days
- CLA : $(T1 - 1.75)$; $(17 - 1.75) = 15.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(41 - 31) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(47 - 45) + 3.5 = 5.5$ days



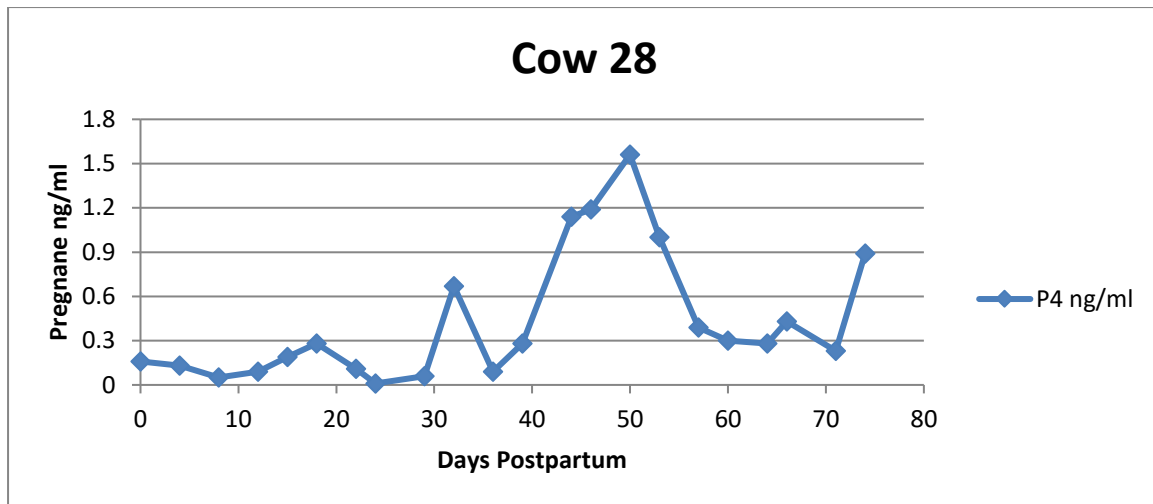
Normal

- First rise of pregnane ($>0.3\text{ng/ml}$) occurred within 45 days of postpartum (day 22; 0.32ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(36 - 22) + 3.5 = 17.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(43 - 39) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(22 - 1.75) = 20.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(57 - 45) + 3.5 = 15.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(65 - 60) + 3.5 = 8.5$ days



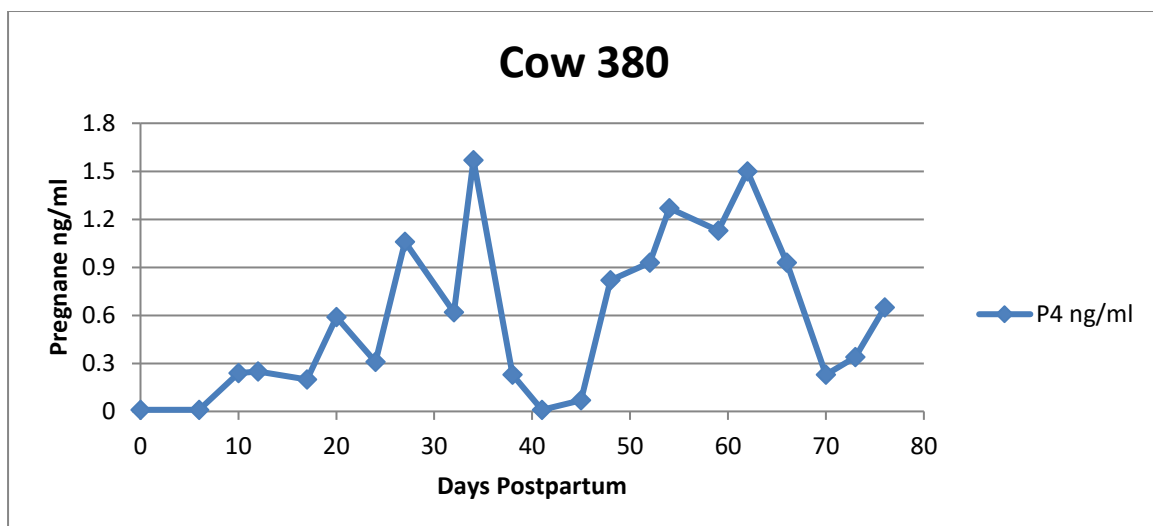
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane ($>0.3\text{ng/ml}$) occurred at day 53 postpartum; 0.33ng/ml .
- Luteal phase : $(T2 - T1 + 3.5)$; $(55 - 53) + 3.5 = 5.5$ days
- Inter-luteal interval : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(53 - 1.75) = 51.25$ days



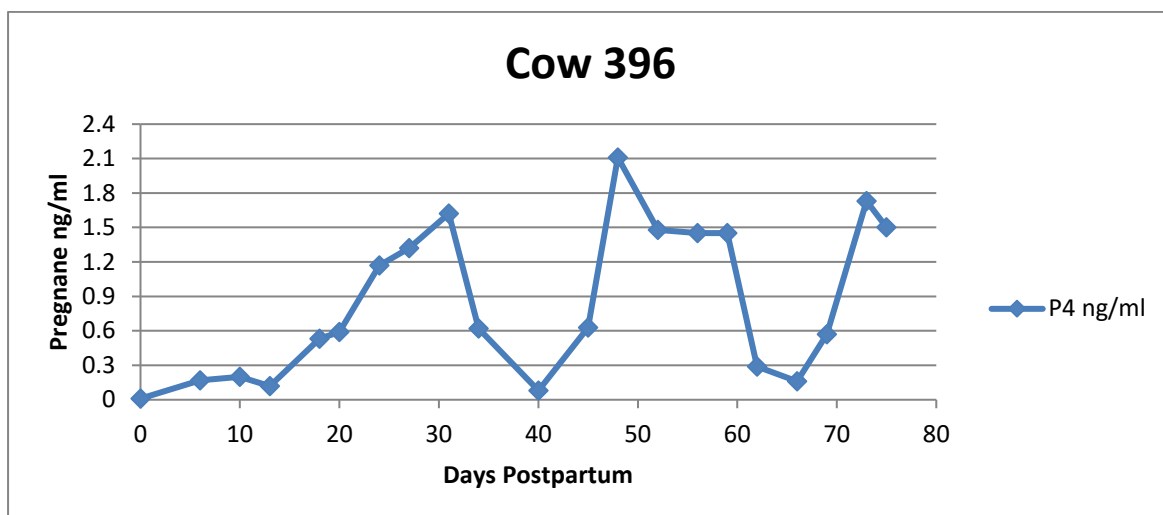
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 32; 0.67ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(39 - 36) + 3.5 = 6.5$ days
- CLA: $(T1 - 1.75)$; $(32 - 1.75) = 30.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(60 - 44) + 3.5 = 19.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



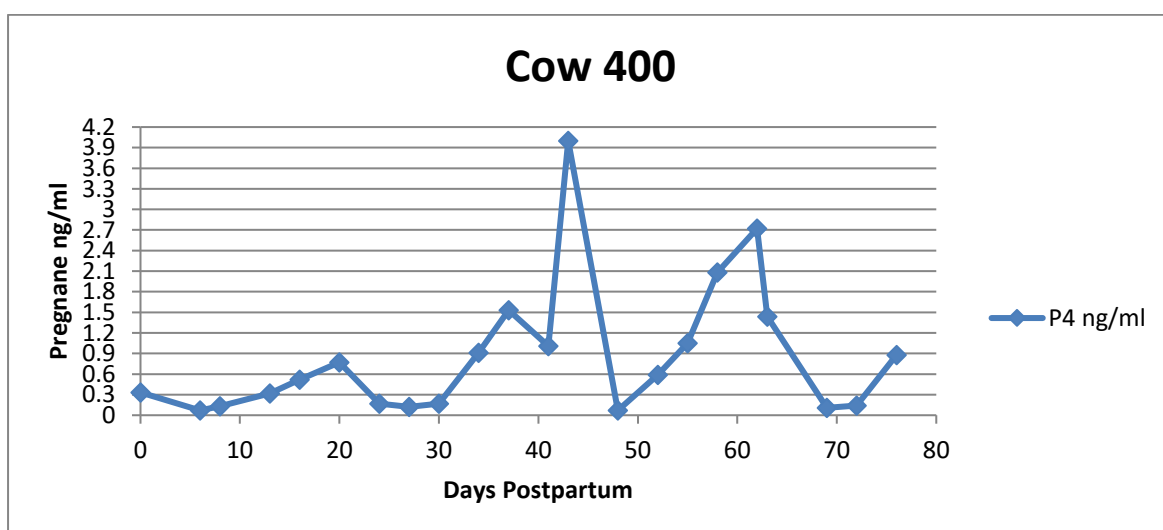
PCL2

- First rise of pregnane (>0.3ng/ml) occurred at day 20 postpartum; 0.59ng/ml.
- 1st LP : $(T2 - T1 + 3.5)$; $(34 - 20) + 3.5 = 17.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(45 - 38) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(20 - 1.75) = 18.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(66 - 48) + 3.5 = 21.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



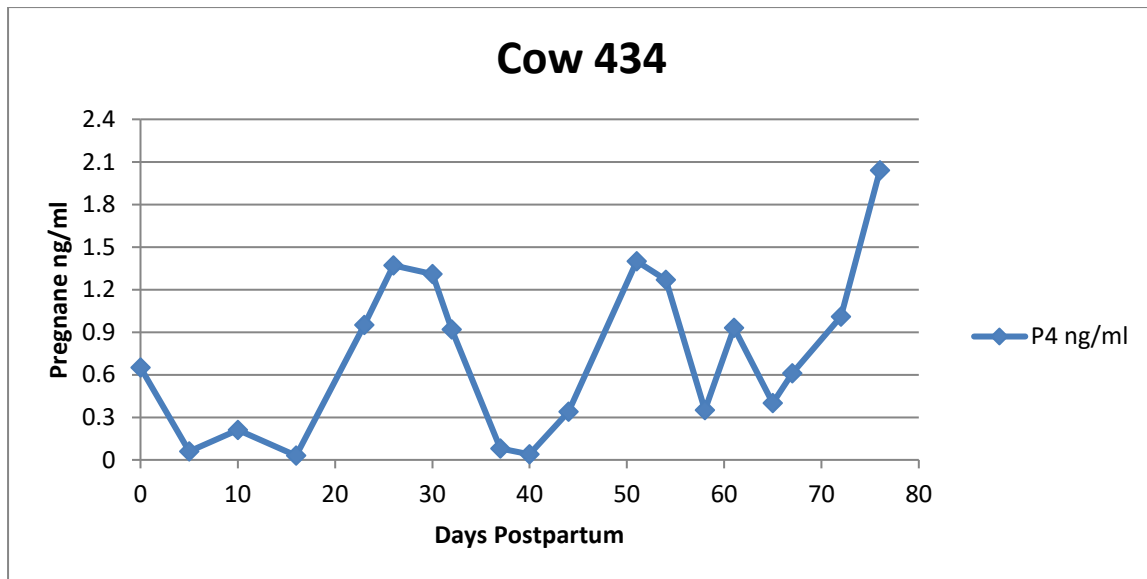
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.53ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(34 - 18) + 3.5 = 19.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(59 - 45) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(66 - 62) + 3.5 = 7.5$ days



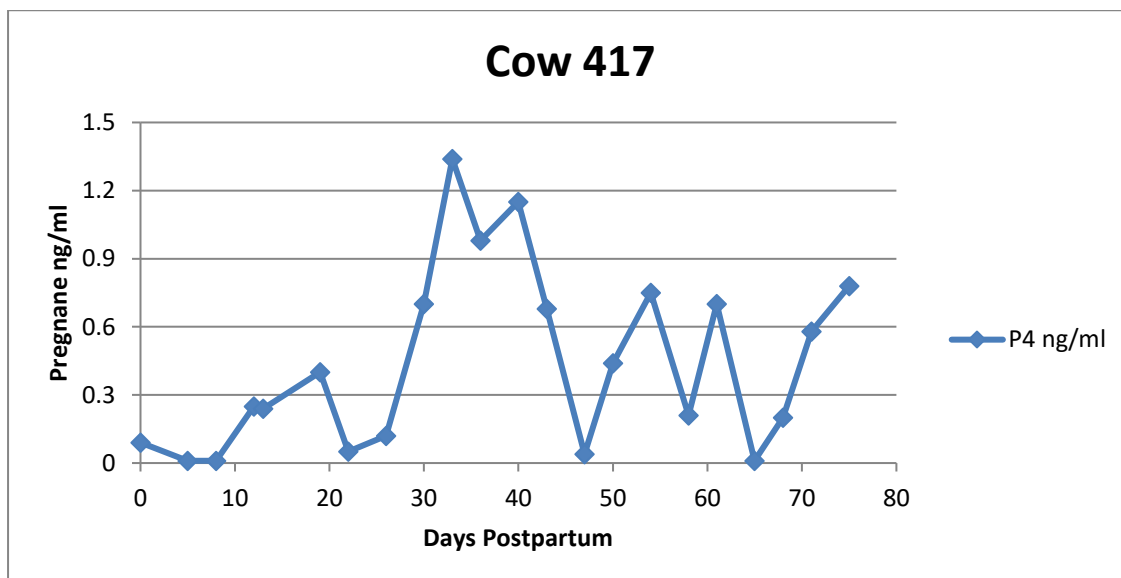
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 13; 0.32ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(20 - 13) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(30 - 24) + 3.5 = 9.5$ days
- CLA : $(T1 - 1.75)$; $(13 - 1.75) = 11.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(43 - 34) + 3.5 = 12.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



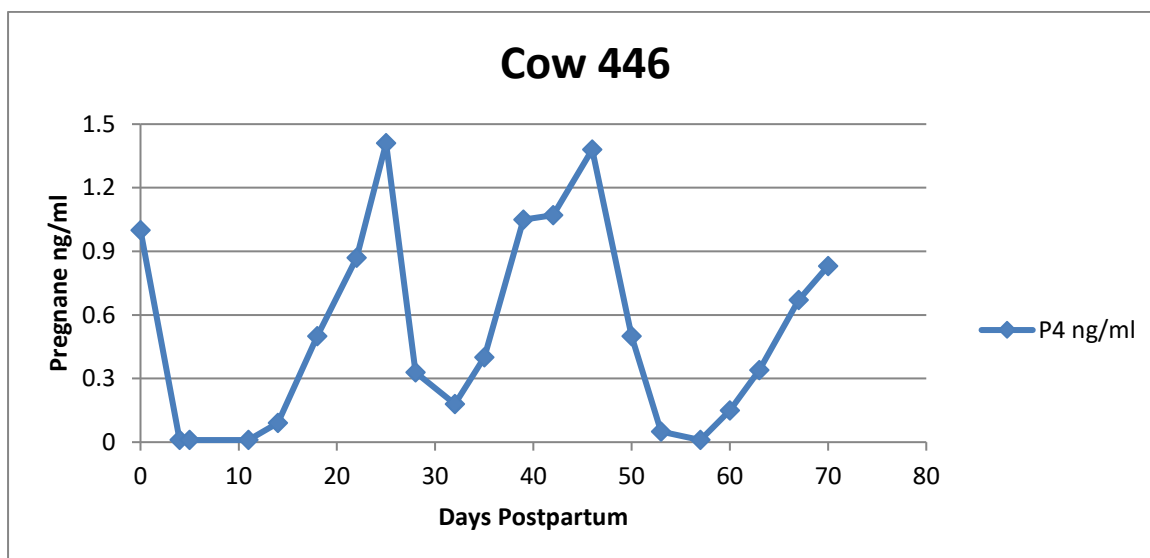
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 0.95ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(32 - 23) + 3.5 = 12.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(40 - 37) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(76 - 44) + 3.5 = 35.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$



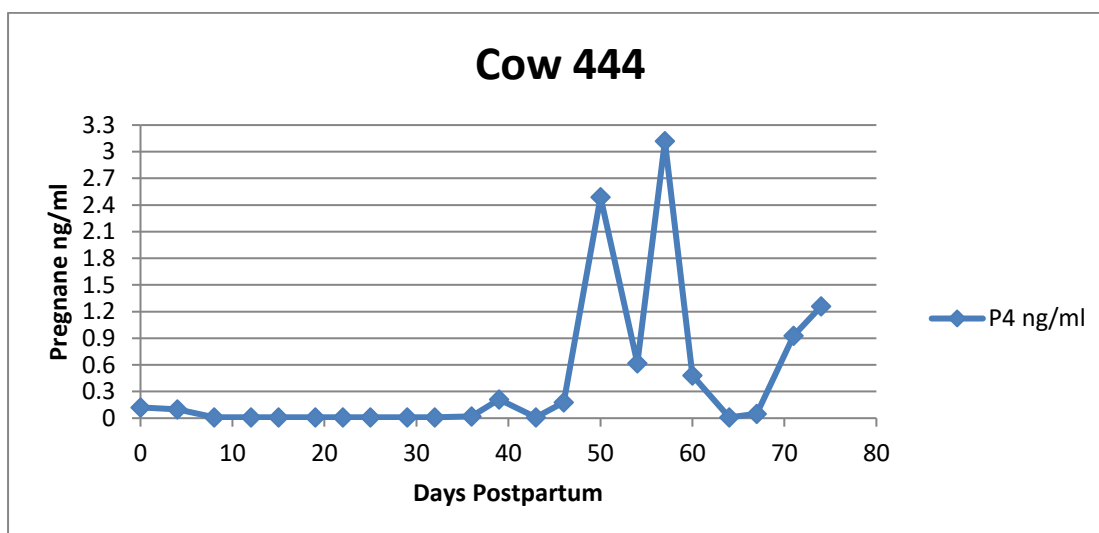
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 19; 0.40ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(26 - 22) + 3.5 = 7.5$ days
- CLA : $(T1 - 1.75)$; $(19 - 1.75) = 17.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(43 - 30) + 3.5 = 16.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



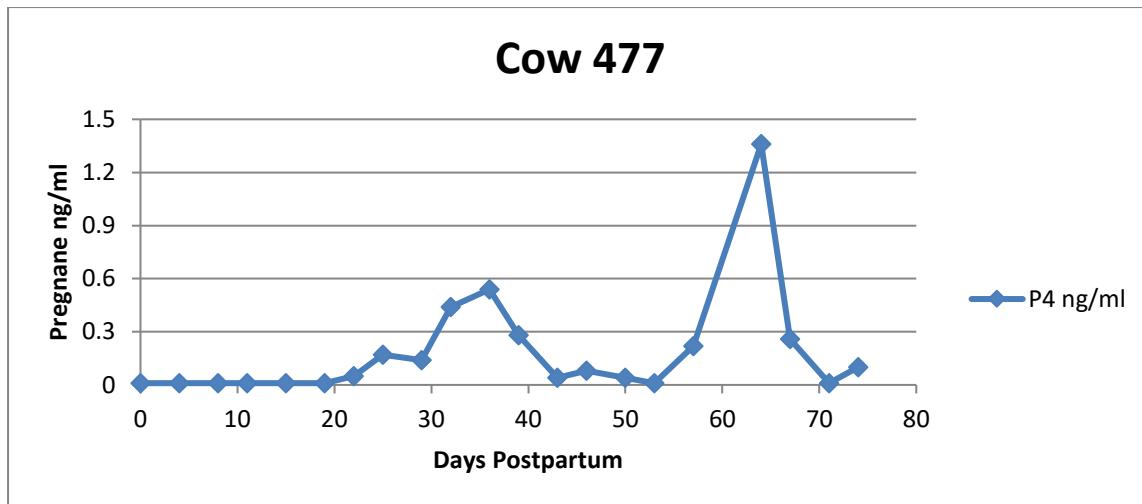
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.50ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(28 - 18) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(50 - 35) + 3.5 = 18.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(60 - 53) + 3.5 = 10.5$ days



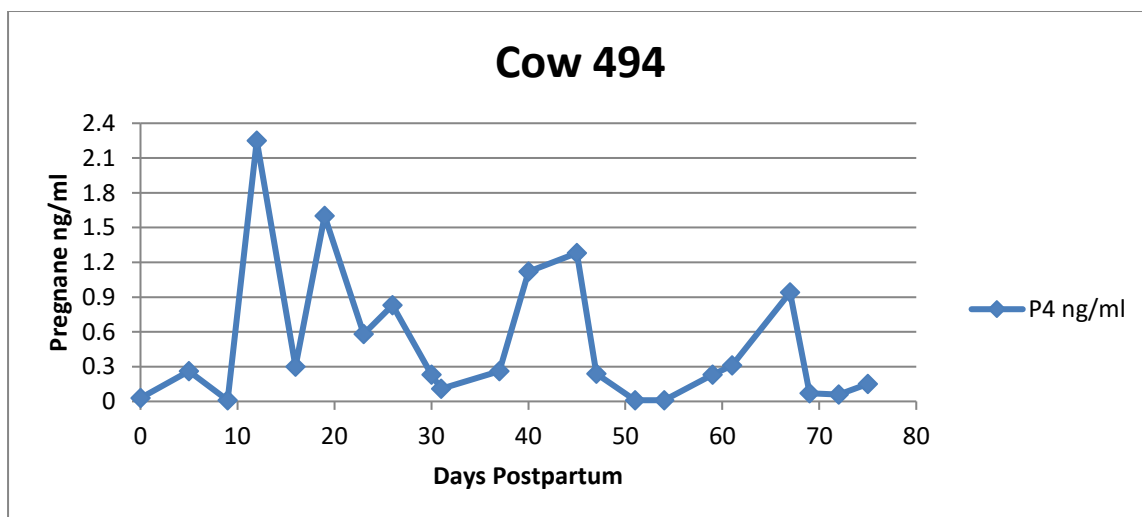
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 50 postpartum; 2.49ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(60 - 50) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(67 - 64) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(50 - 1.75) = 48.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



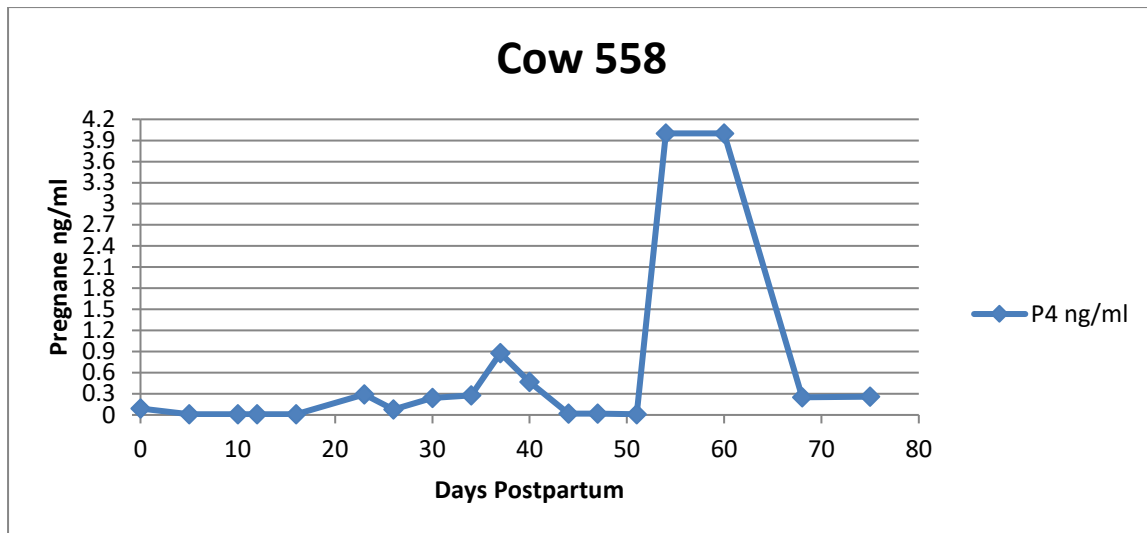
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 32; 0.44ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(36 - 32) + 3.5 = 7.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(57 - 39) + 3.5 = 21.5$ days
- CLA : $(T1 - 1.75)$; $(32 - 1.75) = 30.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(74 - 67) + 3.5 = 10.5$ days



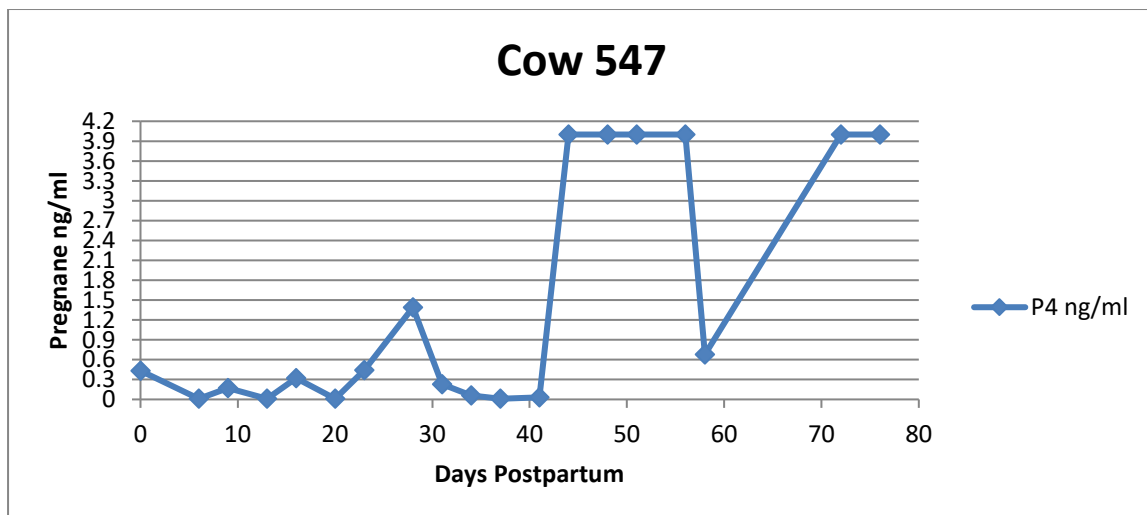
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 12; 2.25ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(26 - 12) + 3.5 = 17.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(37 - 30) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(12 - 1.75) = 10.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(45 - 40) + 3.5 = 8.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(59 - 47) + 3.5 = 15.5$ days



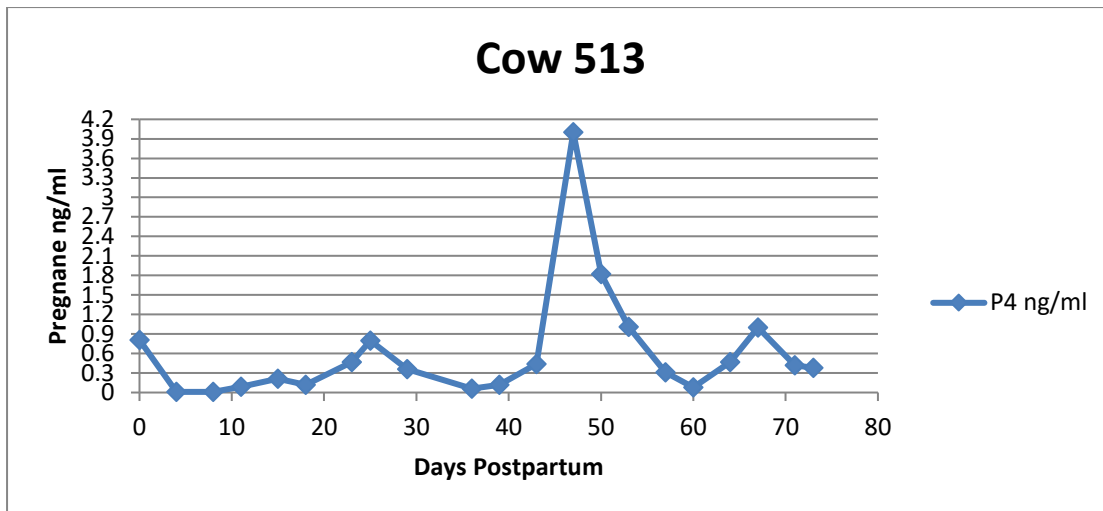
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 37; 0.88ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(40 - 37) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(51 - 44) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(37 - 1.75) = 35.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(60 - 54) + 3.5 = 9.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(75 - 68) + 3.5 = 10.5$ days



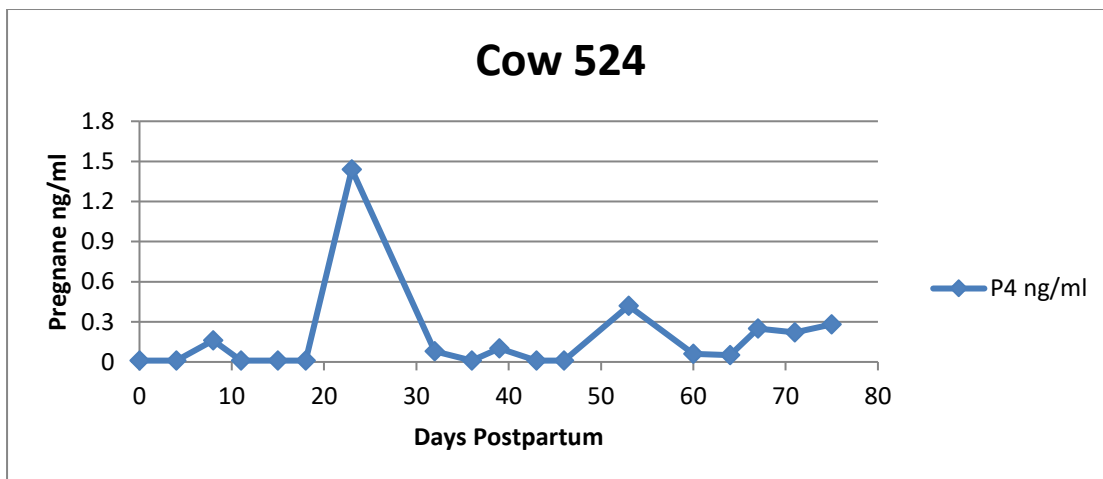
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 16; 0.32ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(16 - 1.75) = 14.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(28 - 23) + 3.5 = 8.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(41 - 31) + 3.5 = 13.5$ day



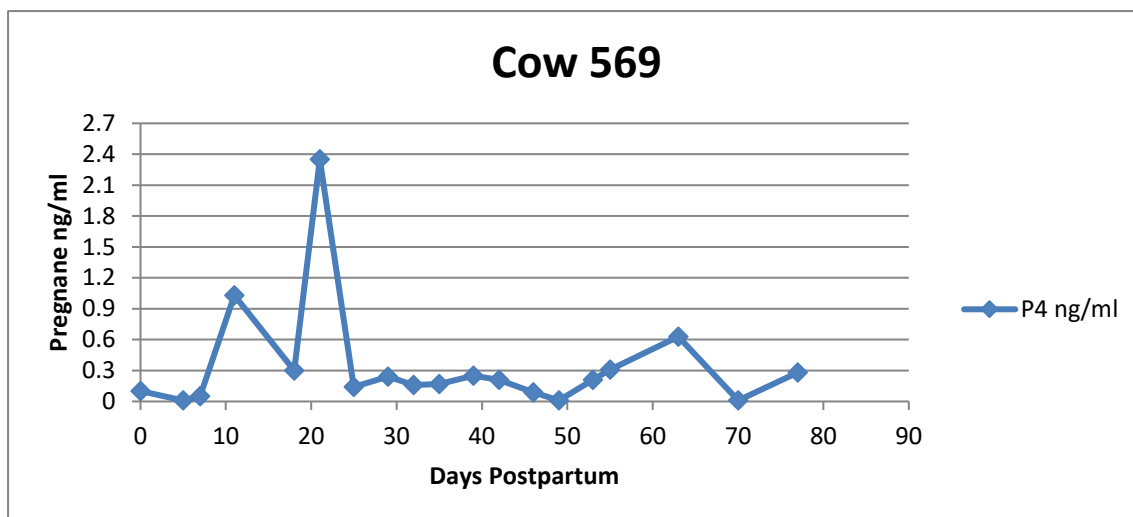
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 0.47ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 23) + 3.5 = 9.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(39 - 36) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(57 - 43) + 3.5 = 17.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



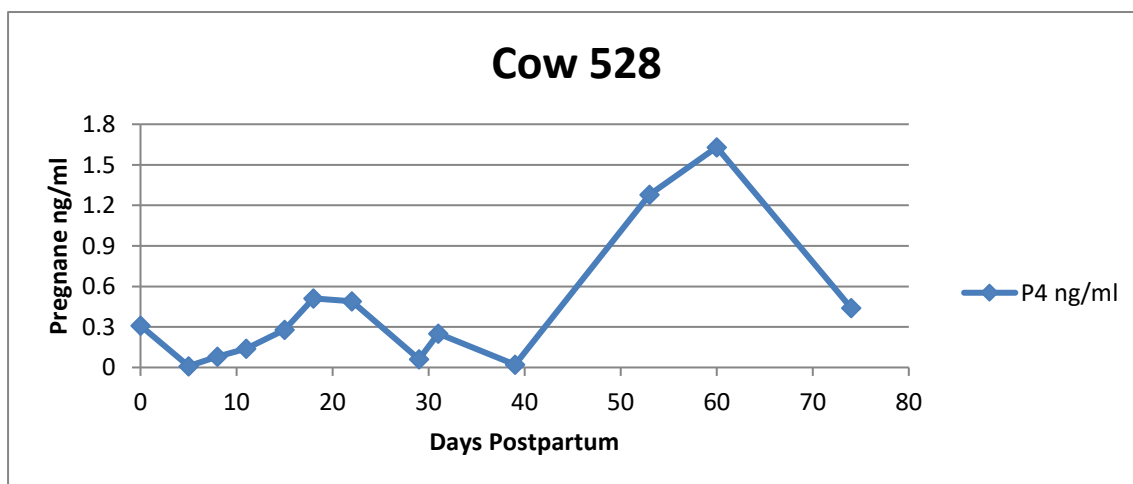
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 1.44ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(46 - 32) + 3.5 = 17.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(75 - 60) + 3.5 = 18.5$ days



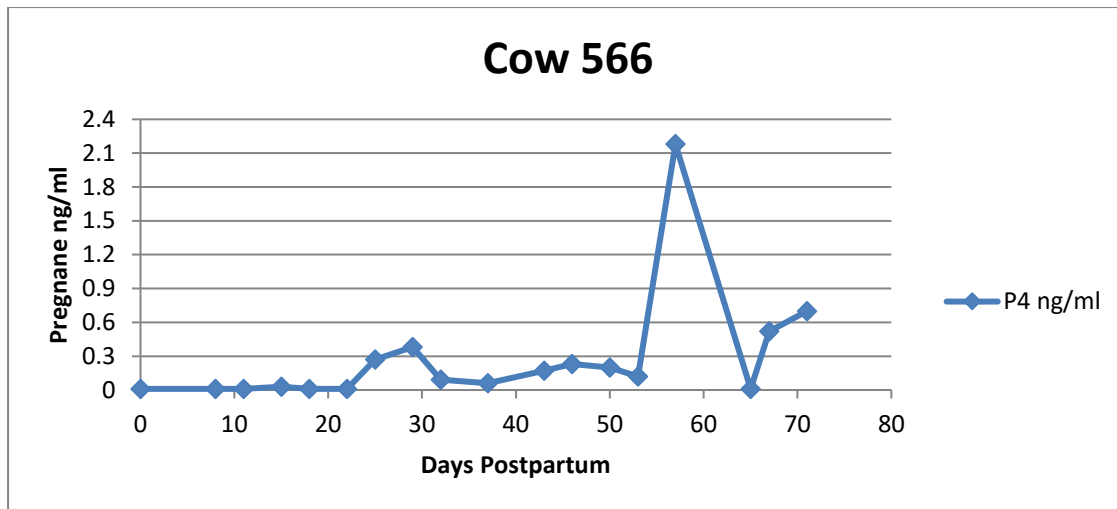
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 11; 1.03ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(21 - 11) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(53 - 25) + 3.5 = 31.5$ days
- CLA : $(T1 - 1.75)$; $(11 - 1.75) = 9.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(63 - 55) + 3.5 = 11.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(77 - 70) + 3.5 = 10.5$ days



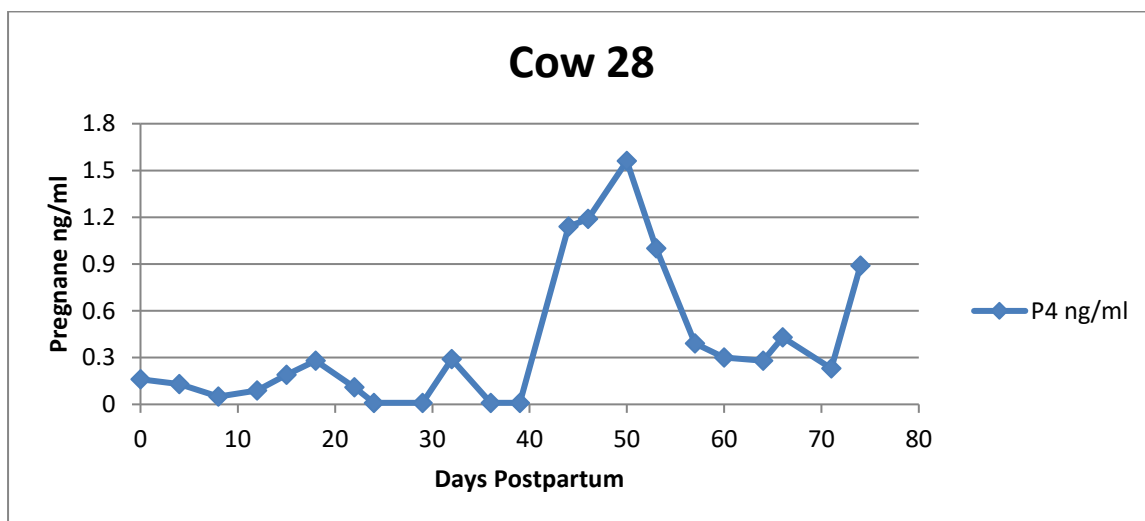
PCL2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.51ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(22 - 18) + 3.5 = 7.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(39 - 29) + 3.5 = 13.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(74 - 53) + 3.5 = 24.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$;



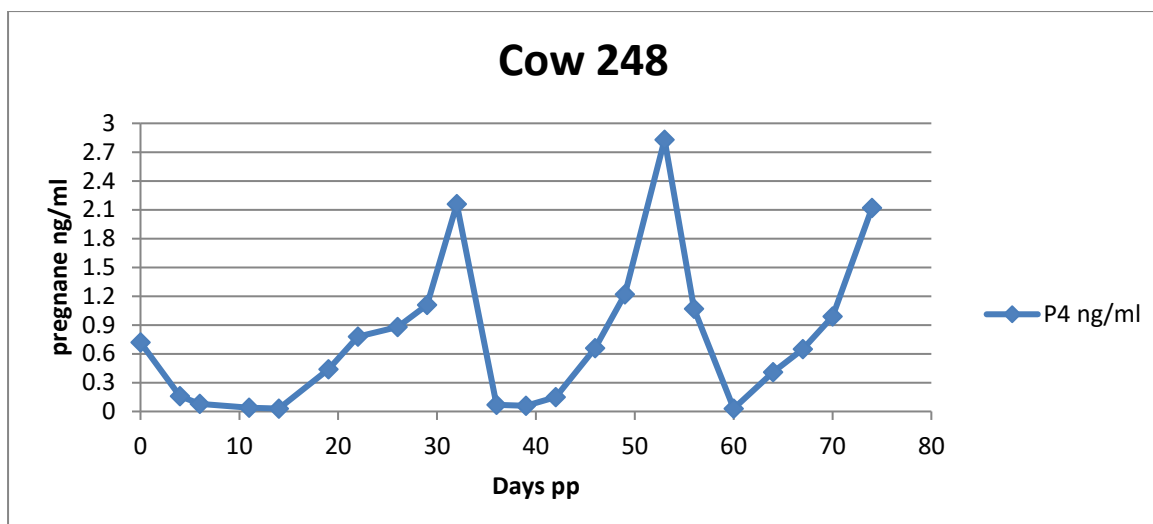
DOV2

- First rise of pregnane (>0.3ng/ml) occurred at day 29 postpartum; 0.38ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(53 - 32) + 3.5 = 24.5$ days
- CLA : $(T1 - 1.75)$; $(29 - 1.75) = 27.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



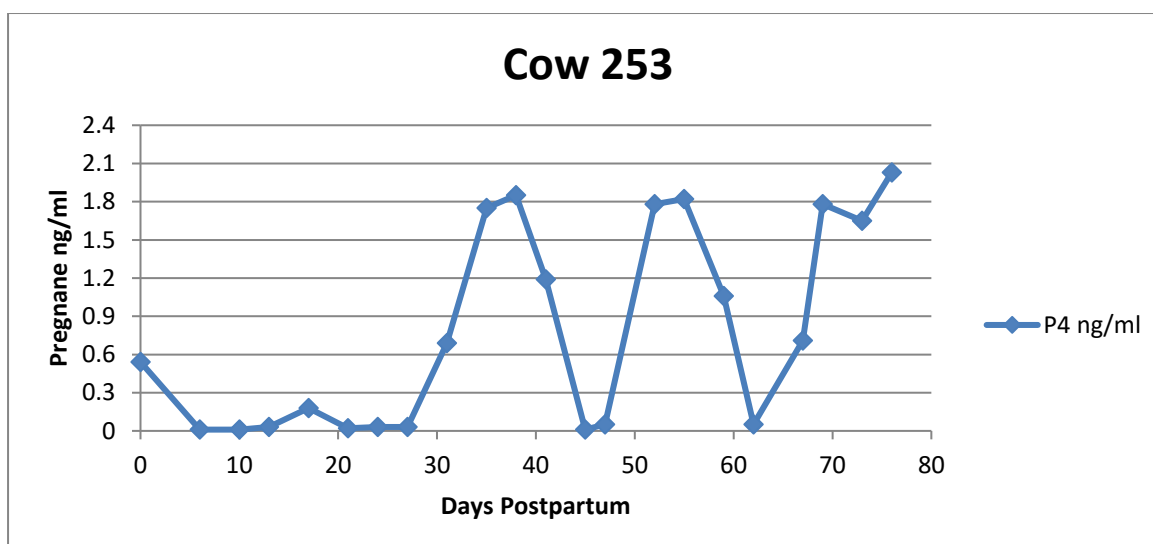
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 44; 1.14ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(60 - 44) + 3.5 = 19.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(44 - 1.75) = 42.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



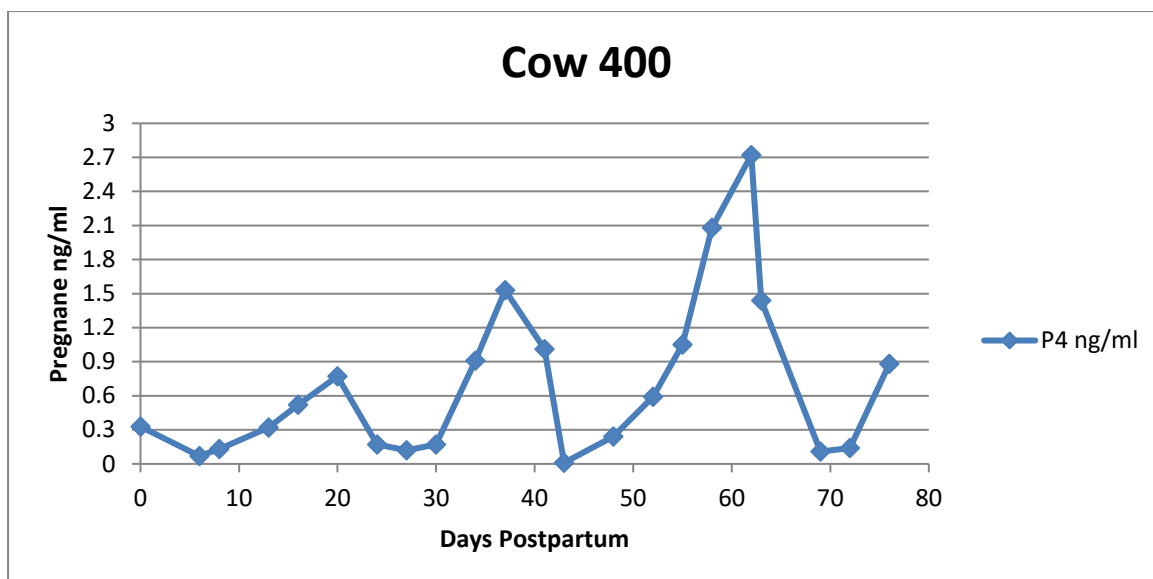
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 19; 0.44ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(32 - 19) + 3.5 = 16.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(42 - 36) + 3.5 = 9.5$ days
- CLA : $(T1 - 1.75)$; $(19 - 1.75) = 17.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(56 - 46) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



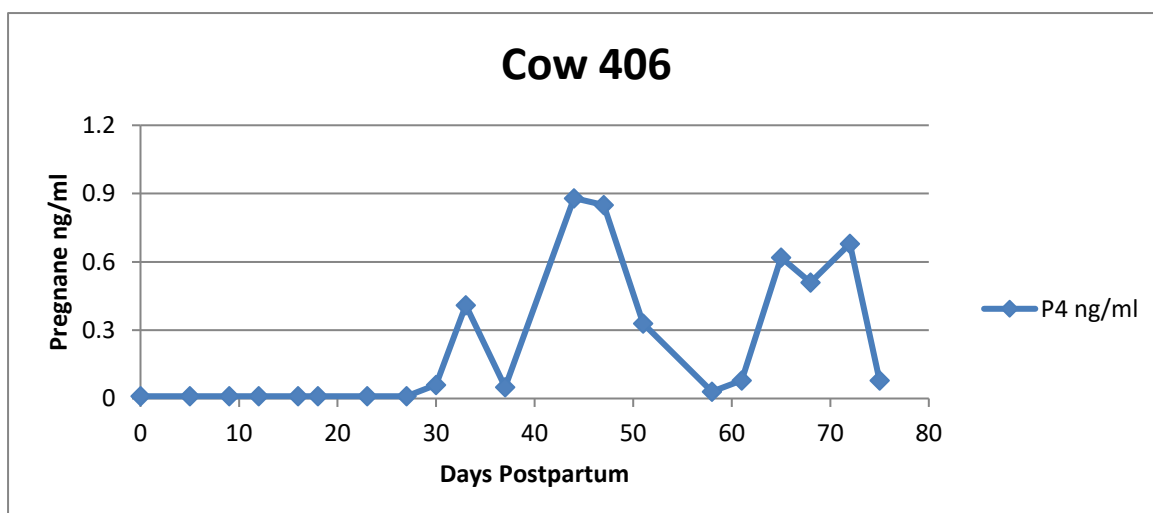
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 31; 0.69ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(41 - 31) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(47 - 45) + 3.5 = 5.5$ days
- CLA : $(T1 - 1.75)$; $(31 - 1.75) = 29.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(59 - 52) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5) = 3.5$ days



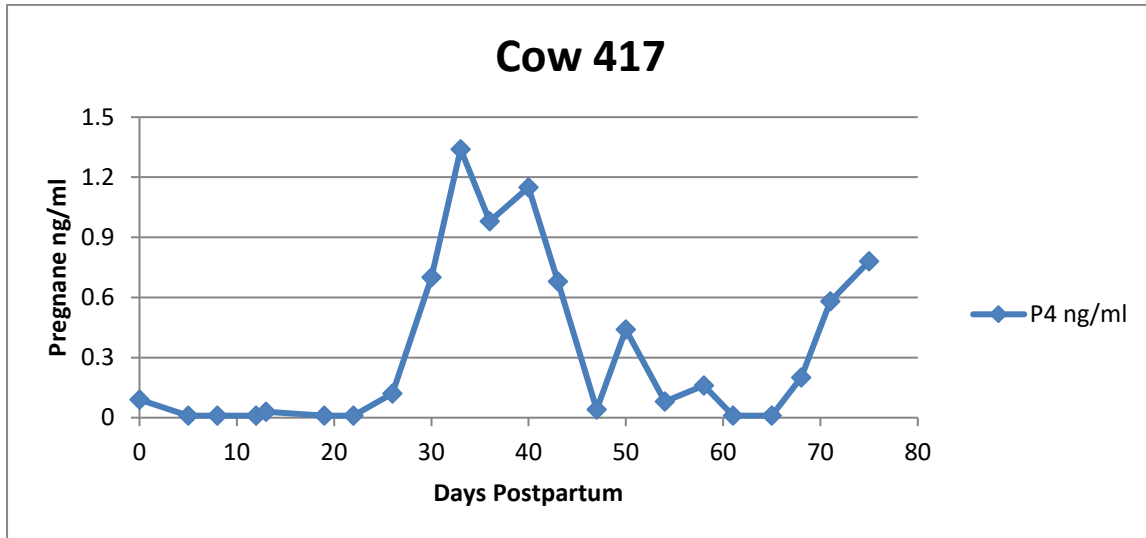
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 13; 0.32ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(20 - 13) + 3.5 = 10.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(30 - 24) + 3.5 = 9.5$ days
- CLA : $(T1 - 1.75)$; $(13 - 1.75) = 11.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(41 - 34) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(48 - 43) + 3.5 = 8.5$ days



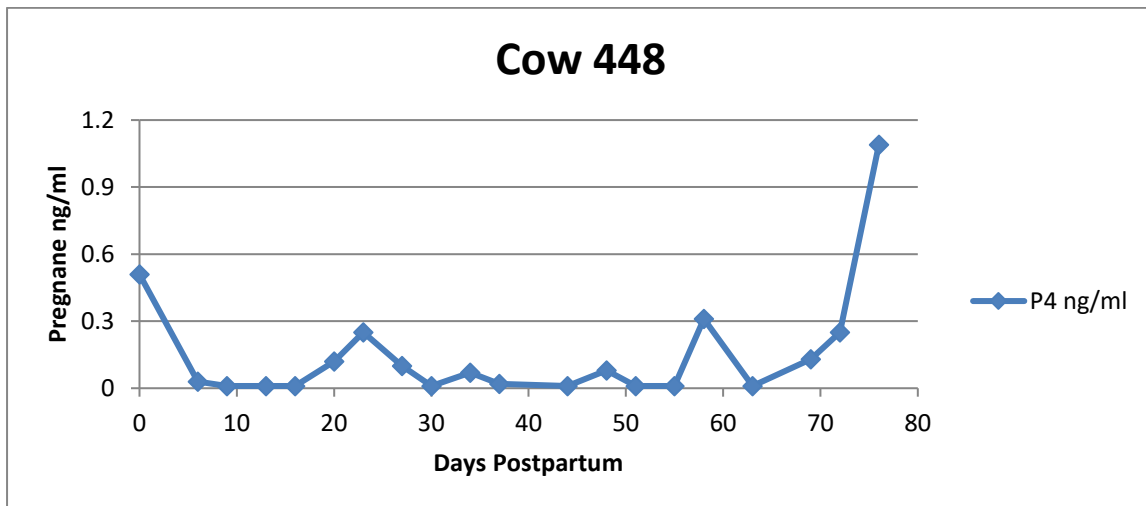
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 33; 0.41ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(33 - 1.75) = 31.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(51 - 44) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(61 - 58) + 3.5 = 6.5$ days



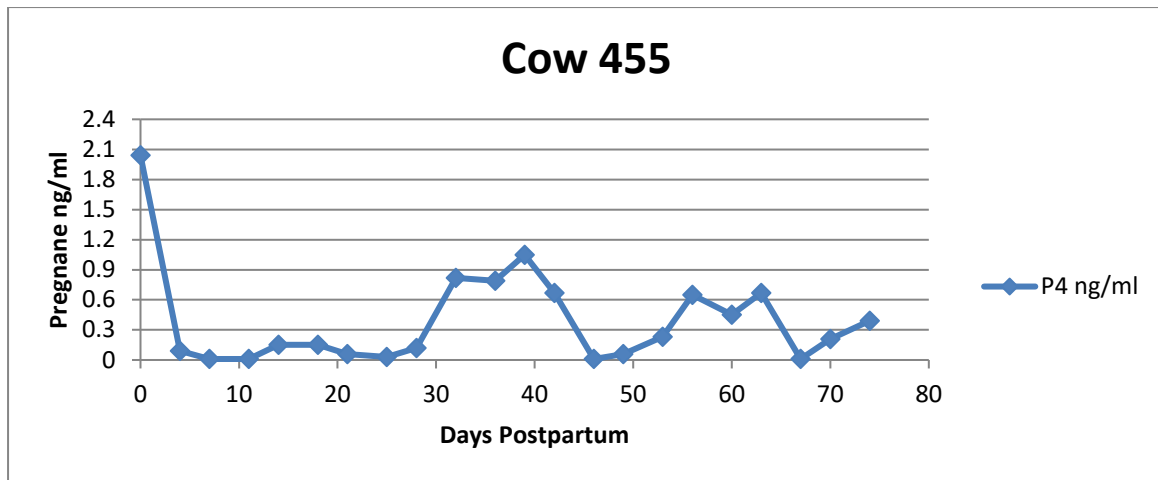
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 30; 0.70ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(43 - 30) + 3.5 = 16.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(30 - 1.75) = 28.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(68 - 54) + 3.5 = 17.5$ days



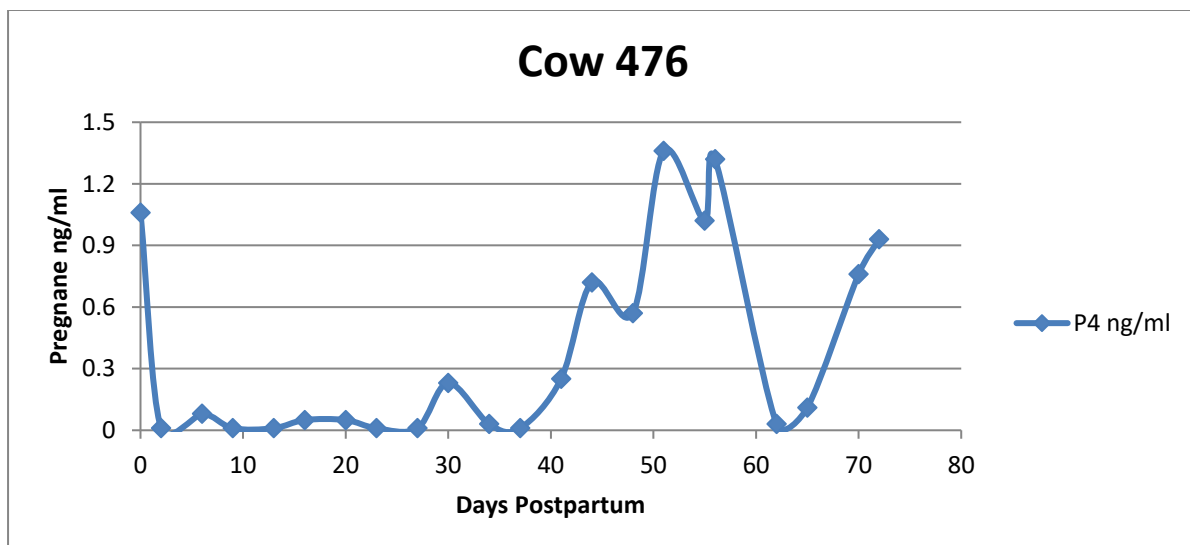
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 58 postpartum; 0.31ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(72 - 63) + 3.5 = 12.5$ days
- CLA : $(T1 - 1.75)$; $(58 - 1.75) = 56.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



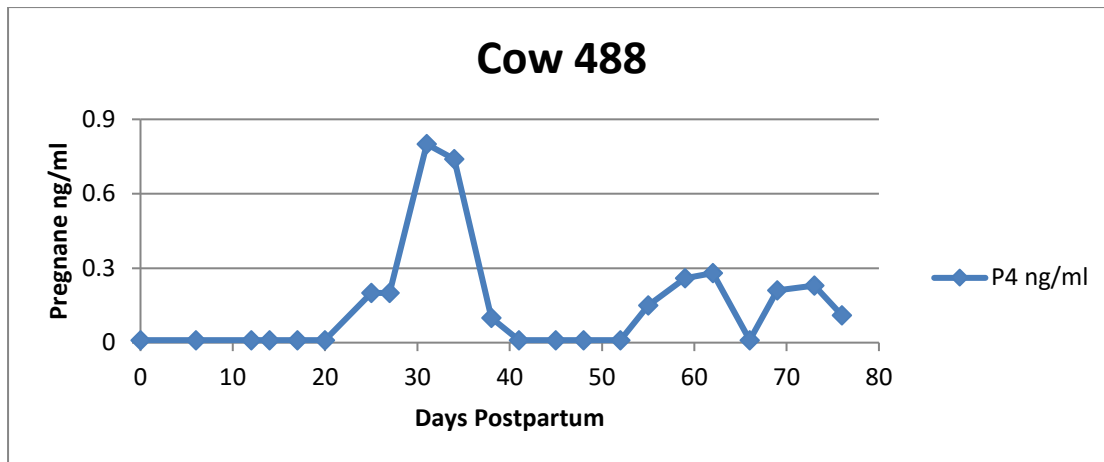
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 32; 0.82ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(42 - 32) + 3.5 = 13.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(53 - 46) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(32 - 1.75) = 30.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(63 - 56) + 3.5 = 10.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(70 - 67) + 3.5 = 6.5$ days



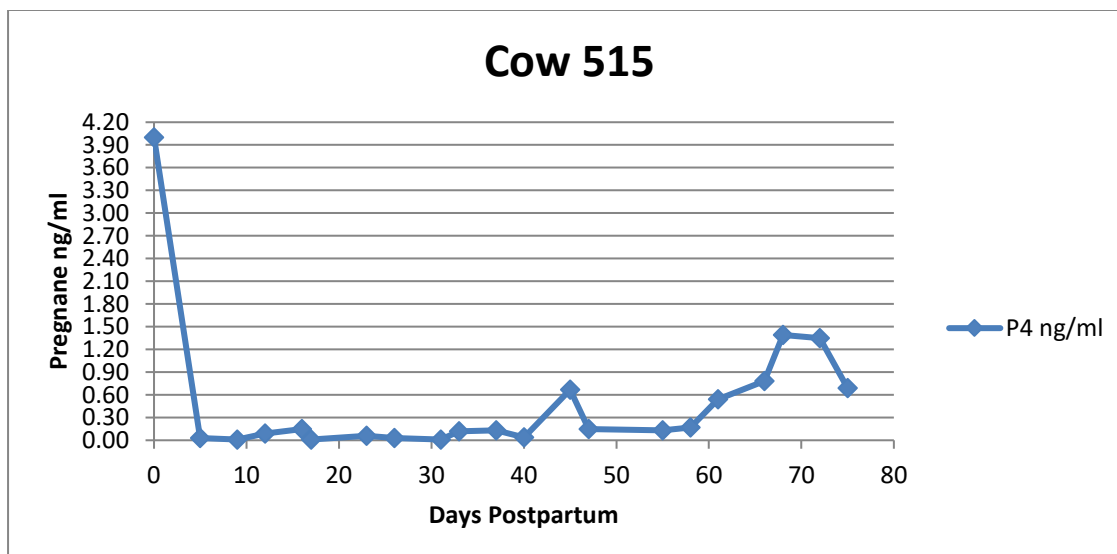
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 44; 0.72ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(56 - 44) + 3.5 = 15.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(65 - 62) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(44 - 1.75) = 42.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



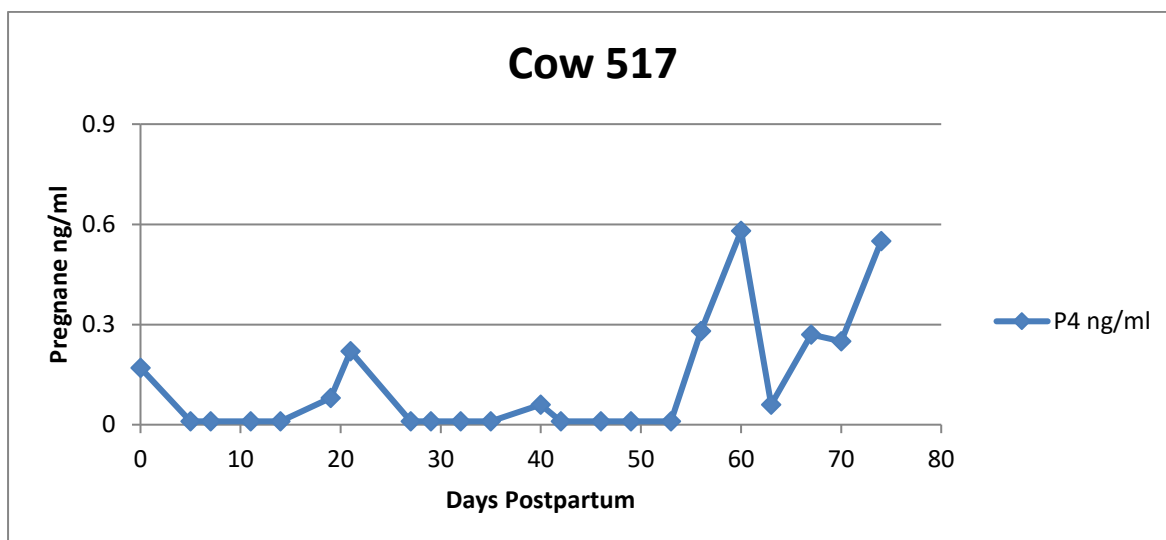
DOV2

- First rise of pregnane (>0.3ng/ml) occurred at day 31 postpartum; 0.80ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(34 - 31) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(76 - 38) + 3.5 = 41.5$ days
- CLA : $(T1 - 1.75)$; $(31 - 1.75) = 29.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



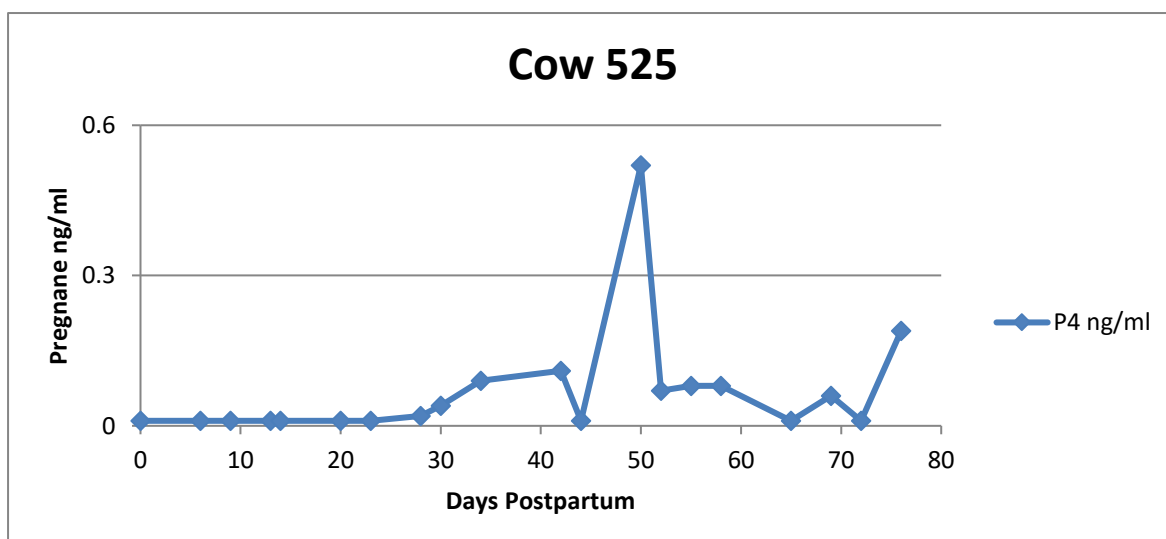
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 45; 0.67ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(58 - 47) + 3.5 = 14.5$ days
- CLA : $(T1 - 1.75)$; $(45 - 1.75) = 43.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



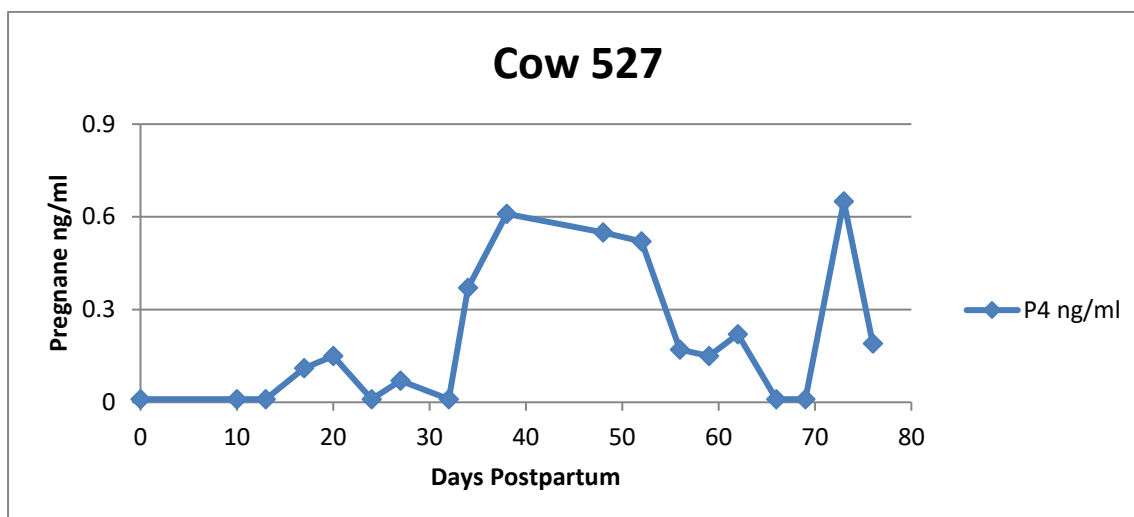
DOV1

- First rise of pregnane (>0.3ng/ml) occurred at day 60 postpartum; 0.58ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(70 - 63) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(60 - 1.75) = 58.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$
- 2nd ILI : $(T6 - T5 + 3.5)$



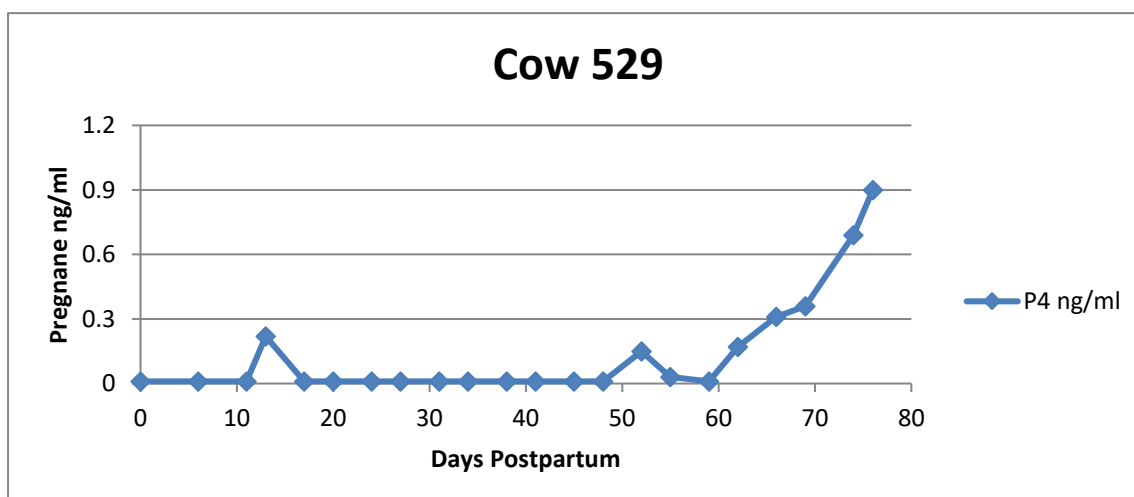
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 50; 0.52ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(76 - 52) + 3.5 = 27.5$ days
- CLA : $(T1 - 1.75)$; $(50 - 1.75) = 48.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



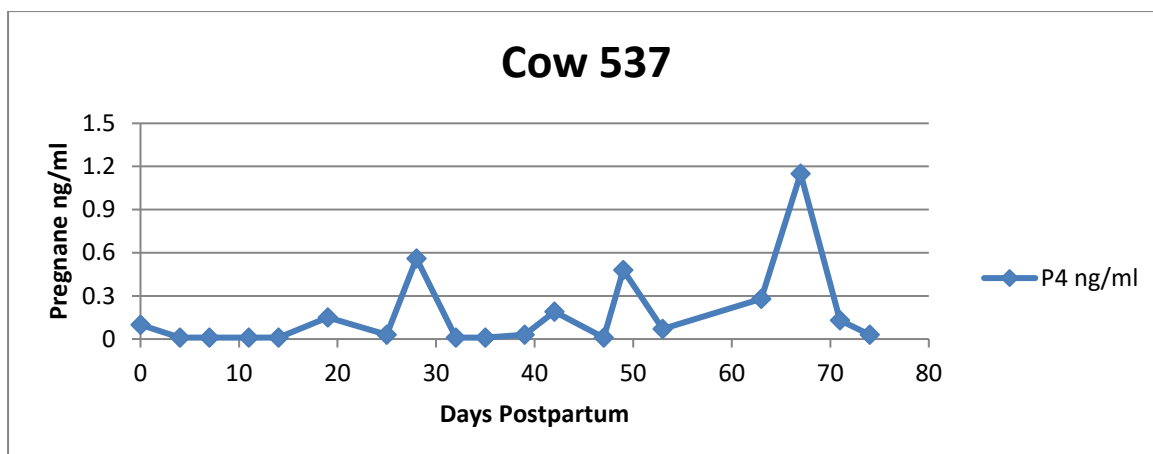
PCL1

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 34; 0.37ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(52 - 34) + 3.5 = 21.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(69 - 56) + 3.5 = 16.5$ days
- CLA : $(T1 - 1.75)$; $(34 - 1.75) = 32.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$



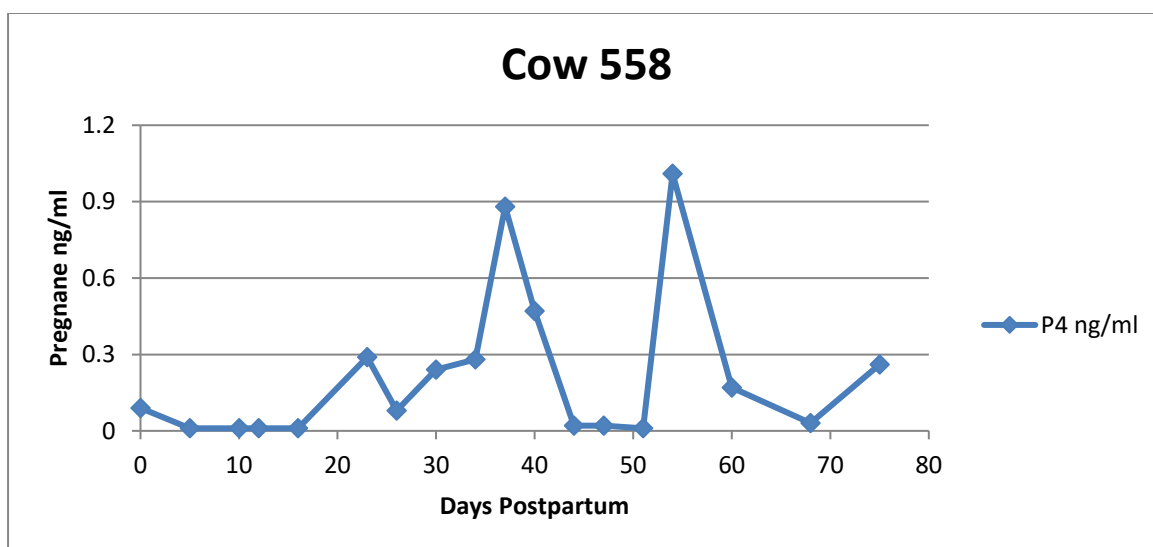
DOV1

- Consistently low pregnane concentrations for >45 days postpartum.
- First rise of pregnane (>0.3ng/ml) occurred at day 66 postpartum; 0.31ng/ml.
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$;
- CLA : $(T1 - 1.75)$; $(66 - 1.75) = 64.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$;
- 2nd ILI : $(T6 - T5 + 3.5)$;



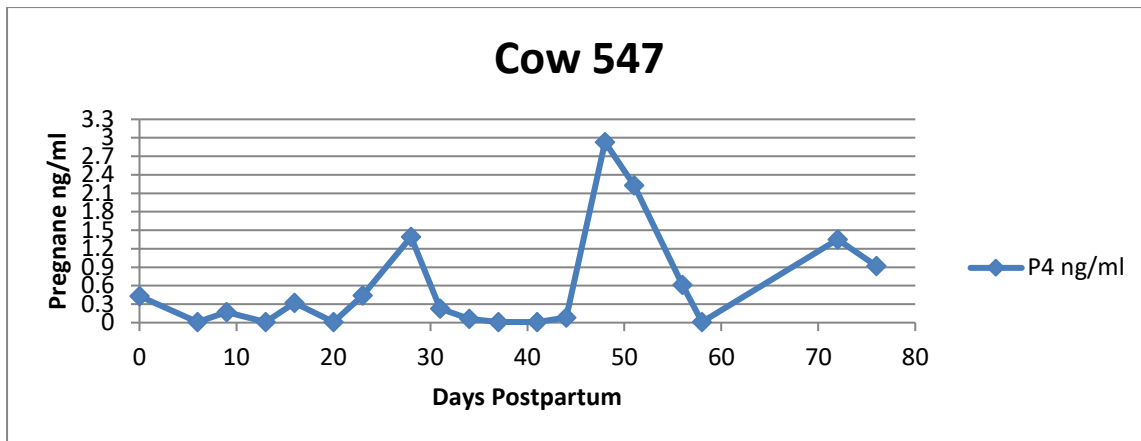
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 28; 0.56ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(47 - 32) + 3.5 = 18.5$ days
- CLA : $(T1 - 1.75)$; $(28 - 1.75) = 26.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(63 - 53) + 3.5 = 13.5$ days



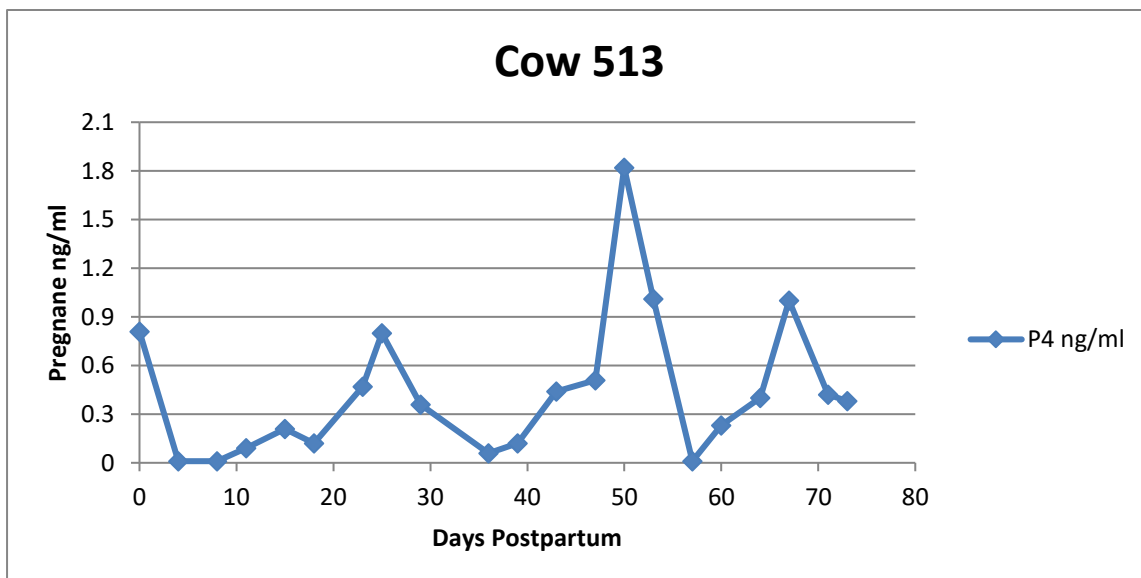
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 37; 0.88ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(40 - 37) + 3.5 = 6.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(51 - 44) + 3.5 = 10.5$ days
- CLA : $(T1 - 1.75)$; $(37 - 1.75) = 35.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(75 - 60) + 3.5 = 18.5$ days



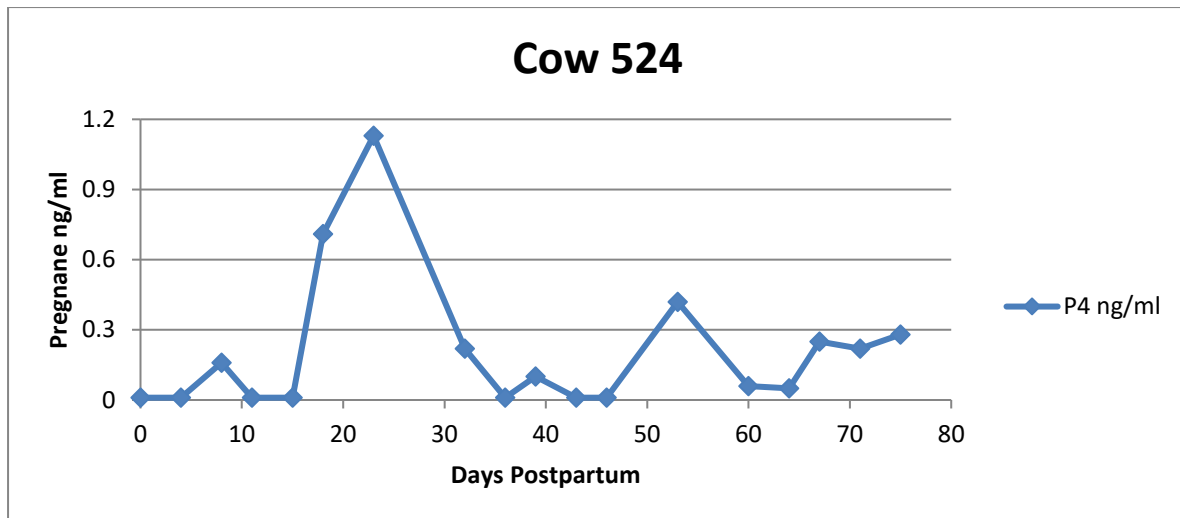
DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 16; 0.32ng/ml).
- 1st LP : $(T2 - T1 + 3.5) = 3.5$ days
- 1st ILI : $(T6 - T5 + 3.5) = 3.5$ days
- CLA : $(T1 - 1.75)$; $(16 - 1.75) = 14.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(28 - 23) + 3.5 = 8.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(44 - 31) + 3.5 = 16.5$ days



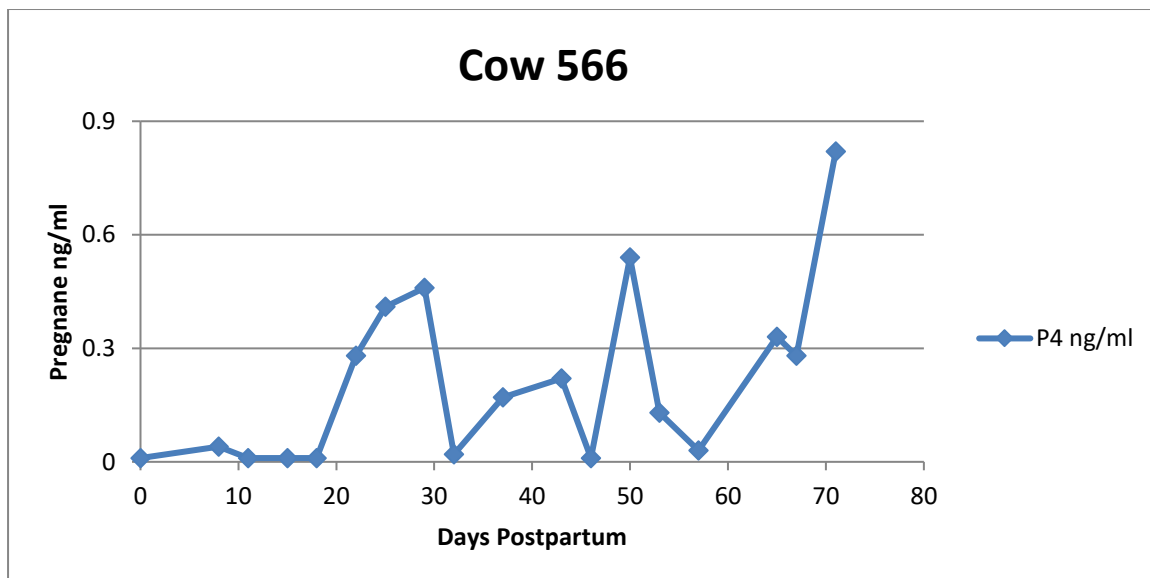
Normal

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 23; 0.47ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 23) + 3.5 = 9.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(39 - 36) + 3.5 = 6.5$ days
- CLA : $(T1 - 1.75)$; $(23 - 1.75) = 21.25$ days
- 2nd LP : $(T2 - T1 + 3.5)$; $(53 - 43) + 3.5 = 13.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(60 - 57) + 3.5 = 6.5$ days



DOV2

- First rise of pregnane (>0.3ng/ml) occurred within 45 days of postpartum (day 18; 0.71ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(23 - 18) + 3.5 = 8.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(46 - 32) + 3.5 = 17.5$ days
- CLA : $(T1 - 1.75)$; $(18 - 1.75) = 16.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(75 - 60) + 3.5 = 18.5$ days



DOV2

- First rise of pregnane (>0.3ng/ml) occurred at day 25 postpartum; 0.41ng/ml).
- 1st LP : $(T2 - T1 + 3.5)$; $(29 - 25) + 3.5 = 7.5$ days
- 1st ILI : $(T6 - T5 + 3.5)$; $(46 - 32) + 3.5 = 17.5$ days
- CLA : $(T1 - 1.75)$; $(25 - 1.75) = 23.25$ days
- 2nd LP : $(T2 - T1 + 3.5) = 3.5$ days
- 2nd ILI : $(T6 - T5 + 3.5)$; $(57 - 53) + 3.5 = 7.5$ days